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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
David K.R.	Karaolis	Baltimore, MD

☐ *Additional inventors are being named on the _____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Methods and Uses of Modulating Microbial Cylic Dinucleotides

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 66 ☐ CD(s), Number

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☐ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.

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Respectfully submitted,

SIGNATURE _____ Date 07/25/2003

TYPED or PRINTED NAME Aaron D. Adams REGISTRATION NO. *(if appropriate)* 50,278

TELEPHONE 410-706-1933 Docket Number: DK-2003-060

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TITLE: **Methods and Uses of Modulating Microbial Cyclic Dinucleotides**

INVENTOR:
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ABSTRACT:
A useful method to regulate the biofilm formation, extracellular polysaccharide, virulence and other phenotypes of prokarytoc and eukaryotic cells including the growth and replication of a species (*in vitro* or *in vivo*) by modulating the level of cyclic dinucleotide (c-di-GMP). Modulation of c-diGMP levels can be by direct exogenous addition of c-di-GMP, by adding analogs of c-di-GMP, or by the manipulation of c-di-GMP cyclases (and inhibitors) and c-di-GMP phosphodiesterases (and inhibitors) that affect synthesis or degradation of c-di-GMP, respectively.

FIELD OF THE INVENTION:
This invention relates to the use of modulating the levels of cyclic dinucleotides (and analogs), cyclic dinucleotide cyclase and cyclic dinucleotide phosphodiesterase activity, in order to regulate various phenotypes of prokaryotic and eukaryotic cells. The invention proposes a method to inhibit and control microbial colonization, virulence and infections caused by a wide variety of microbial species, including promoting therapy of antibiotic resistant biofilm-associated microbes, microbial colonization of catheters and indwelling devices, reducing microbial biofilms in industrial pipelines and reducing microbial growth on building and construction materials. The method inhibits biofilm formation, development and promotes biofilm dissolution. The method can be used to control or inhibit a variety of bacterial species, including but not limited to: Vibrio harveyi, Vibrio cholerae, Vibrio parahaemolyticus, Vibrio alginolyticus, Pseudomonas fluorescens, Pseudomonas aeruginosa, Pseudomonas acidovorans, Pseudomonas alcaligenes, Pseudomonas putida, Pseudomonas syringae, Pseudomonas aureofaciens, Pseudomonas fragi, Fusobacterium nucleatum, Treponema denticola, Citrobacter freundii, Porphyromonas gingivalis, Moraxella catarrhalis, Stenotrophomonas maltophilia, Burkholderia cepacia, Aeromonas hydrophilia, Salmonella typhi, Salmonella paratyphi, Salmonella Enteritidis, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Enterobacter cloacae, Enterobacter aerogenes Yersinia enterocolitica, Yersinia pestis, Yersinia pseudotuberculosis, Yersinia intermedia, Bordetella pertussis, Bordetella parapertussis, Bordetella bronchiseptica, Escherichia coli, Salmonella typhimurium, Haemophilus influenzae, Haemophilus parainfluenzae, Haemophilus haemolyticus, Haemophilus parahaemolyticus, Pasteurella multocida, Pasteurella haemolytica, Gardnerella vaginalis, Bacteroides spp., Clostridium difficile, Mycobacterium avium, Mycobacterium intracellulare, Mycrobacterium leprae, Corynebacterium diphtheriae, Corynebacterium ulcerans, Legionella pneumophila, Listeria monocytogenes Helicobacter pylori, Bacillus subtilis, Bacillus anthracis, Borrelia burgfdorferi, Neisseria meningitidis, Neisseria gonorrhoeae, Borrelia burgdorferi, Campylobacter fetus, Campylobacter jejuni, Campylobacter coli,, Deinococcus radiodurans, Mycobacterium tuberculosis, Desulfvibrio spp., Actinomyces spp., Erwinia spp., Xanthomonas spp., Xylella spp., Clavibacter spp., Desulfomonas spp., Desulfovibrio spp., Desulfococcus spp., Desulfobacter spp., Desulfobulbus spp., Desulfosarcina spp., Deslfuromonas spp., Acinetobacter calcoaceticus, Acinetobacter haemolyticus, Enterococcus faecalis, Streptococcus

pneumoniae, Streptococcus pyogenes, Streptococcus agalactiae, Staphylococcus aureus, Staphylococcus epidermidis, Klebsiella pneumoniae, Klebsiella oxytoca, Serratia marcescens, Francisella tularensis, Morganella morganii, Providencia alcalifaciens, Providencia rettgeri, Providencia stuartii,, Proteus mirabilis, Proteus vulgaris, Streptomyces spp., Clostridium spp., Rhodococcus spp., Thermatoga spp., Sphingomonas spp., Zymomonas spp., Micrococcus spp., Azotobacter spp., Norcardia spp., Brevibacterium spp., Alcaligenes spp., Microbispora spp., Micromonospora spp., Methylobacterium organophilum, Pseudomonas reptilivora, Pseudomonas carragienovora, Pseudomonas dentificans, Corynebacterium spp., Propionibacterium spp., Xanothomonas spp., Methylobacterium spp., Chromobacterium spp., Saccharopolyspora spp., Actinobacillus spp., Alteromonas spp., Aeronomonas spp., Agrobacterium tumefaciens, Staphylococcus aureus, Staphylococcus epidennidis, Staphylococcus hominis, Staphylococcus haemolyticus, Staphylococcus warneri, Staphylococcus cohnii, Staphylococcus saprophyticus, Staphylococcus capitis, Staphylococcus lugdunensis, Staphylococcus intemedius, Staphylococcus hyicus, Staphylococcus saccharolyticus and Rhizobium spp., and mutant derivatives thereof.

The method can be used to control or inhibit a variety of fungal species, including but not limited to: Absidia spp., Actinomadura madurae, Actinomyces spp., Allescheria boydii, Alternaria spp., Anthopsis deltoidea, Aphanomyces spp., Apophysomyces eleqans, Armillaria spp., Arnium leoporinum, Aspergillus spp., Aureobasidium pullulans, Basidiobolus ranarum, Bipolaris spp., Blastomyces dermatitidis, Botrytis spp., Candida spp., Centrospora spp., Cephalosporium spp., Ceratocystis spp., Chaetoconidium spp., Chaetomium spp., Cladosporium spp., Coccidioides immitis, Colletotrichum spp, Conidiobolus spp., Corynebacterium tenuis, Cryptoporiopsis spp., Cylindrocladium spp., Cryptococcus spp., Cunninghamella bertholletiae, Curvularia spp., Dactylaria spp., Diplodia spp., Epidermophyton spp., Epidermophyton floccosum, Exserophilum spp., Exophiala spp., Fonsecaea spp., Fulvia spp., Fusarium spp., Geotrichum spp., Guignardia spp., Helminthosporium spp., Histoplasma spp., Lecythophora spp., Macrophomina spp., Madurella spp., Magnaporthe spp., Malassezia furfur, Microsporum spp., Monilinia spp., Mucor spp., Mycocentrospora acerina, Nectria spp., Nocardia spp., Oospora spp., Ophiobolus spp., Paecilomyces spp., Paracoccidioides brasiliensis, Penicillium spp., Phaeosclera dematioides, Phaeoannellomyces spp., Phialemonium obovatum, Phialophora spp., Phlyctaena spp., Phoma spp., Phomopsis spp., Phymatotrichum spp., Phytophthora spp., Pythium spp., Piedraia hortai, Pneumocystis carinii, Puccinia spp., Pythium insidiosum, Rhinocladiella aquaspersa, Rhizomucor pusillus, Rhizoctonia spp., Rhizopus spp., Saccharomyces spp., Saksenaea vasiformis, Sarcinomyces phaeomuriformis, Scerotium spp., Sclerotinia spp., Sphaerotheca spp., Sporothrix schenckii, Syncephalastrum racemosum, Stachybotrys chartarum, Taeniolella boppii, Taphrina spp., Thielaviopsis spp., Torulopsosis spp., Trichophyton spp., Trichosporon spp., Ulocladium chartarum, Ustilago spp., Venturia spp., Verticillium spp., Wangiella dermatitidis, Whetxelinia spp., Xylohypha spp., and their synonyms.

CLAIMS:

1. Modulating microbial cyclic dinucleotide (and analogs) levels to affect and reduce microbial colonization.
2. Modulating microbial cyclic dinucleotide levels (and analogs) to affect and reduce microbial virulence.
3. Modulating microbial cyclic dinucleotide levels (and analogs) to affect and reduce microbial exopolysaccharide.
4. Modulating microbial cyclic dinucleotide levels (and analogs) to affect and reduce microbial motility.
5. Modulating microbial cyclic dinucleotide levels (and analogs) to affect and reduce microbial biofilm formation.
6. Modulating microbial cyclic dinucleotide phosphodiesterase levels to affect microbial colonization.
7. Modulating microbial cyclic dinucleotide phosphodiesterase levels to affect microbial virulence.
8. Modulating microbial cyclic dinucleotide phosphodiesterase levels to affect and reduce microbial exopolysaccharide.
9. Modulating microbial cyclic dinucleotide phosphodiesterase levels to affect and reduce microbial motility.
10. Modulating microbial cyclic dinucleotide phosphodiesterase levels to affect and reduce microbial biofilm formation.
11. Modulating microbial cyclic dinucleotide cyclase levels to affect microbial colonization.
12. Modulating microbial cyclic dinucleotide cyclase levels to affect microbial virulence.
13. Modulating microbial cyclic dinucleotide cyclase levels to affect and reduce microbial exopolysaccharide.
14. Modulating microbial cyclic dinucleotide cyclase levels to affect and reduce microbial motility.
15. Modulating microbial cyclic dinucleotide cyclase levels to affect and reduce microbial biofilm formation.
16. Construction and use of a microbial cyclic dinucleotide phosphodiesterase mutant that is altered in phosphodiesterase activity and cyclic dinucleotide levels such that colonization, virulence and biofilm forming ability is affected.
17. Construction and use of a microbial cyclic dinucleotide cyclase mutant that is altered in cyclase activity and cyclic dinucleotide levels such that colonization, virulence and biofilm forming ability is affected.
18. A method of preventing and treating bacterial infections comprising administering a physiologically effective dose of an inhibitor of microbial cyclic dinucleotide phosphodiesterase.
19. A method of preventing and treating bacterial infections comprising administering a physiologically effective dose of an inhibitor of microbial cyclic dinucleotide cyclase.
20. A method for treating infections by exopolysaccharide (EPS)-producing bacteria by modulating microbial cyclic dinucleotides.
21. A method for treating infections by modulating microbial cyclic dinucleotide phosphodiesterase.
22. A method for treating infections by modulating microbial cyclic dinucleotide cyclase.

23. Engineered microbial vectors for the delivery of cyclic dinucleotide phosphodiesterase inhibitors.
24. Engineered microbial vectors for the delivery of cyclic dinucleotide cyclase inhibitors.
25. Non-microbial delivery of cyclic dinucleotide phosphodiesterase inhibitors.
26. Non-microbial delivery of cyclic dinucleotide cyclase inhibitors.
27. Engineered vectors for the delivery of agents modulating cyclic dinucleotides.
28. Non-microbial delivery of agents modulating microbial cyclic dinucleotides.
29. Use of agents that modulate cyclic dinucleotides in combination with antimicrobial agents.
30. Use of agents that modulate cyclic dinucleotides in combination with therapeutic agents.
31. Use of agents that modulate cyclic dinucleotides in combination with a vaccine component.
32. Use of agents that modulate cyclic dinucleotide phosphodiesterase activity in combination with antimicrobial agents.
33. Use of agents that modulate cyclic dinucleotide phosphodiesterase or cyclase activity in combination with therapeutic agents.
34. Use of agents that modulate microbial cyclic dinucleotide phosphodiesterase or cyclase activity in combination with a vaccine component.
35. Use of agents that modulate microbial cyclic dinucleotides prior, during or after microbial infection.
36. Use of agents that modulate microbial cyclic dinucleotide phosphodiesterase or cyclase prior, during or after microbial infection.
37. Use of agents that modulate eukaryotic cyclic dinucleotides prior, during or after microbial infection.
38. Use of agents that modulate eukaryotic cyclic dinucleotide phosphodiesterase or cyclase prior, during or after microbial infection.
39. Use of a microbial cyclic dinucleotide cyclase mutant as a live attenuated vaccine strain.
40. Use of a microbial cyclic dinucleotide phosphodiesterase mutant as a live attenuated vaccine strain.
41. An attenuated strain of a bacteria, said bacteria comprising altered cyclic dinucleotide phosphodiesterase or cyclase activity such that bacteria are attenuated.
42. The attenuated strain, wherein the altered activity is obtained by a deletion in a cyclic dinucleotide phosphodiesterase or cyclase gene.
43. The attenuated strain, wherein the altered activity is obtained by an increase in cyclic dinucleotide phosphodiesterase or cyclase expression.
44. The attenuated strain, wherein the altered activity is obtained by an artificially engineered change in a genome of a wild-type pathogenic bacteria.
45. The attenuated strain, wherein the change in the bacteria's genome is a change selected from the group consisting of a deletion, an insertion and a mutation of a native sequence.
46. The attenuated strain, wherein the altered activity is obtained by a heterologous nucleotide inserted into a wild-type pathogenic bacteria.
47. The attenuated strain, wherein the heterologous nucleotide is operatively inserted into a plasmid and expresses cyclic dinucleotide phosphodiesterase or cyclase.
48. The attenuated (cyclic dinucleotide phosphodiesterase or cyclase) strain, wherein the bacterial strain is an attenuated form of a pathogenic bacteria.

49. A method, comprising the steps of: administering to a subject capable of generating an immune response a composition comprising a pharmaceutically acceptable excipient an immunogenic dose of altered bacteria with altered cyclic dinucleotide phosphodiesterase activity which bacteria are attenuated; and allowing the composition to remain in the subject for a time and under conditions to allow the subject to generate an immune response to the bacteria and produce antibodies specific to the bacteria.
50. The above method, whercin the antibodies generated are IgG type antibodies.
51. The method, wherein the IgG antibodies are highly specific for an antigen of the bacteria.
52. The method, wherein the bacteria remain in the subject under conditions and for a period of timesufficient to allow for B cells of the subject to undergo isotype switching and further for the B cells to undergo clonal expansion.
53. The method, wherein an amount of antibodies produced by the subject exceeds 150% of an amount of antibodies which would be produced by the subject administered unaltered bacteria in amount equivalent to the immunogenic dose of altered bacteria.
54. Cloning and expression of cyclic dinucleotide cyclase genes or any components or products thereof for the purpose of modulating microbial cyclic dinucleotide and microbial colonization, exopolysaccharide, virulence and biofilm formation.
55. Cloning and expression of cyclic dinucleotide phosphodiesterase genes or any components or products thereof for the purpose of modulating microbial cyclic dinucleotide levels and affecting microbial colonization, exopolysaccharide, virulence and biofilm formation.
56. Use of cyclic dinucleotide phosphodiesterase inhibitors or any components or products thereof as part of a vaccine.
57. Use of agents that modulate cyclic dinucleotide phosphodiesterase activity as part of a vaccine.
58. Use of agents that modulate microbial cyclic dinucleotides as part of a vaccine.
59. Use of cyclic dinucleotide phosphodiesterase inhibitors or any components or products thereof in an antibiotic preparation.
60. Use of agents that modulate cyclic dinucleotide phosphodiesterase activity or any components or products thereof in an antibiotic preparation.
61. Use of agents that modulate microbial cyclic dinucleotides or any components or products thereof in an antibiotic preparation.

Background

Cholera and *Vibrio cholerae*

Cholera is an important diarrheal disease of humans that results in significant morbidity and mortality (44, 61). Cholera affects more than 75 countries and every continent (Communicable Disease Surveillance and Response, World Health Organization, www.who.org). A total of 293,121 cholera cases and 10,586 deaths were reported to WHO in 1998 (54). Cholera is acquired by drinking fecally contaminated food or water containing pathogenic *Vibrio cholerae*. Because of its high death-to-case ratio, transmissibility, persistence in the environment and its ability to occur in explosive epidemic form, *V. cholerae* is a public health concern. Furthermore, because of the potential threat of weaponized *V. cholerae* to the food and water supply, it is a priority organism in biodefense research. The threat to the economy, environment and human health is also highlighted by the finding that *V. cholerae* has the potential to be transported internationally and invade new regions through the ballast water of ships (47). *V. cholerae* is known to persist in the environment, however, the factors promoting the environmental persistence of *V. cholerae* are not well understood.

V. cholerae can change its phenotype and reversibly switch from EPSoff (smooth colony morphology) to EPSon (rugose colony morphology) in which the cells are embedded in exopolysaccharide (EPS) and display a wrinkled "rugose" colony morphology and an associated biofilm; (2, 50, 78, 84, 85). Biofilms are the primary mode of existence of most bacterial species and are central to cell survival and persistence (15, 18, 22, 77). The switch to EPSon and the rugose phenotype clearly promotes biofilm formation (50, 63, 78). Importantly, EPS is essential for *V. cholerae* biofilm formation (78). The rugose variant is highly chlorine resistant and shows increased resistance to killing by acid, UV light and complement-mediated serum bactericidal activity (50, 63, 85). Therefore, it has been proposed that switching to EPSon and the rugose phenotype is important in *V. cholerae* and promotes survival in various environments (78, 85). Thus, while the rugose phenotype appeared to be important in *V. cholerae*, studies of *V. cholerae* EPS and the rugose variant were impeded by the low frequency of switching to the rugose phenotype under available conditions. It is now becoming increasingly recognized that the rugose phenotype might have an important role in *V. cholerae* and several other species suggesting that these variants might represent the "tip of an iceberg". The rugose or wrinkled colony phenotype consisting of aggregating cells has been reported in *S. enterica* Enteritidis (59), *S. enterica* Typhimurium, (4), *V. parahaemolyticus* (35), *P. aeruginosa* (57) and *Enterobacter sakazakii* (24). Data is accumulating suggesting that rugose variants are filling a specific role in biofilm formation, particular niches or in particular environments. However, the molecular basis of switching to the rugose phenotype is not well understood.

Importance of Biofilms

Since the introduction of antibiotics, bacterial pathogens have proved remarkably effective at developing resistance. Nosocomial infections result in substantive increases in health care costs, length of hospitalization, and morbidity and mortality (65, 69, 71). Most recently, attention has focused on the emergence of multi-antibiotic resistant pathogens as a cause of such infections; such microorganisms are becoming endemic in U.S. hospitals, further exacerbating already severe problems with nosocomial infections. Gram-negative bacilli are frequently associated with nosocomial infections in ICU patients, particularly ventilator-associated pneumonia and catheter-associated urinary tract infections (Table 1) (27). Of particular concern are nosocomial infections caused by enterobacteria-producing extended-spectrum β-lactamases (ESBLs), particularly *K. pneumoniae*. Organisms that possess these enzymes are usually resistant to multiple antibiotics rendering many currently available potent antimicrobials

useless (64). Evaluation of data from NNIS hospitals shows a dramatic increase in the proportion of *K. pneumoniae* isolates that are resistant to ceftriaxone, cefotaxime, or ceftazidime over the last decade, with a much higher increase particularly among isolates recovered from ICU patients (Fig. 1, Table 2). As such, patients who receive care in ICUs are at increased risk for nosocomial infections, especially pneumonia, urinary tract infection, and bloodstream infection (27). The emergence of antibiotic resistant pathogens in ICUs has made treating infections very difficult, and in some cases, impossible. The duration of stay in the hospital, especially in the ICU, has been associated with the acquisition of ESBL-producing *K. pneumoniae* (12, 60, 70) and has been implicated in inter-facility transmission within a geographic region (49).

TABLE 1. Eight most common pathogens associated with nosocomial infection in an ICU patient, National Nosocomial Infections Surveillance System, January 19879-July 1998

	Relative % by Site of Infection				
All sites Pathogen	BSI n=235,758	Pneu n=50,091	UTI n=64,056	SSI n=47,502	n=22,043
S. epidermidis	14.3	39.3	2.5	3.1	13.5
S. aureus	11.4	10.7	16.8	1.6	12.6
P. aeruginosa	9.9	3.0	16.1	10.6	9.2
Enterococci	8.1	10.3	1.9	13.8	14.5
Enterobacter	7.3	4.2	10.7	5.7	8.8
E. coli		7.0	2.9	4.4	18.2 7.1
C. albicans	6.6	4.9	4.0	15.3	4.8
K. pneumoniae	4.7	2.9	6.5	6.1	3.5
Others	30.7	21.8	37.1	25.6	26
Total	100	100	100	100	100

BSI= laboratory confirmed (primary) bloodstream infection; Pneu= pneumonia; UTI= urinary tract infection; SSI= surgical site infection. Adapted from Fridken *et al.* (27).

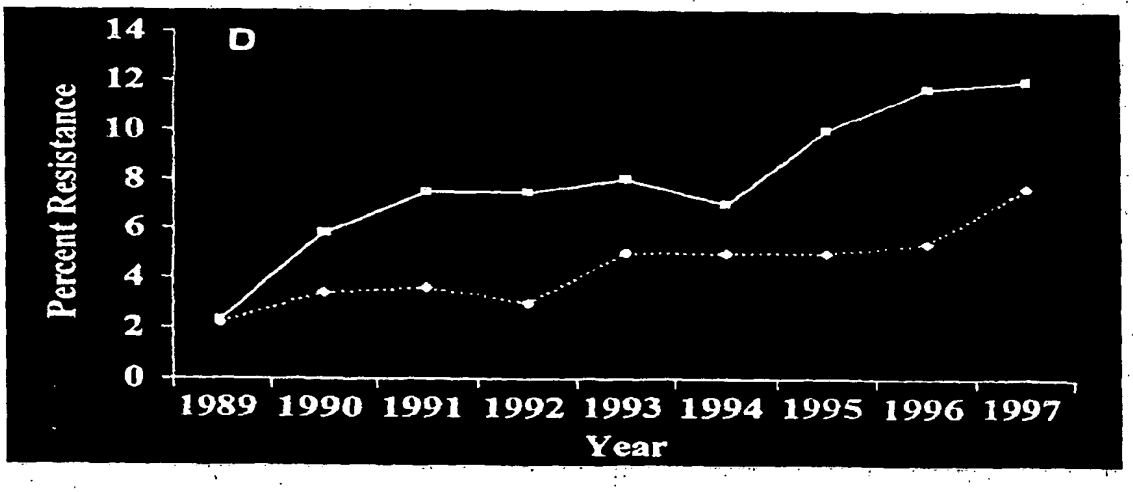


FIGURE 1. Proportion of isolates associated with a nosocomial infection among ICU (solid line) or non-ICU (dotted line) pateinets who were *K. pneumoniae* resistant to third-generation cephalosporins (e.g. ceftriaxone, cefotaxime, or ceftazidime.

TABLE 2. Relative risk of isolating the specific antimicrobial-resistant pathogen from a nosocomial infection occurring in an ICU patient compared with other patients, NNIS, January 1989-1998.

Pathogen	Antimicrobial resistance	Relative Risk among ICU patients (95% CI)*
S. epidermidis	Methicillin	1.22 (1.21-1.24)
S. aureus	Methicillin	1.09 (1.07-1.16)
Enterococci	Vancomycin	1.16 (1.13-1.20)
Enterobacter	Third-generation cephalosprins	1.11 (1.09-1.13)
K. pneumoniae	Third-generation cephalosprins	1.24 (1.20-1.30)
P. aeruginosa	Imipenem	1.16 (1.13-1.21)
P. aeruginosa	Third-generation cephalosprins	1.13 (1.11-1.16)
P. aeruginosa	Ciproflaxacin/ofloxacin	1.03 (1.00-1.05)

* Data from NNIS system, common relative risk and 95% confidence interval, by Mantel-Haenszel Statistic, controlling for year of infection.

Biofilms are the primary mode of existence of many bacterial species and are central to their survival, persistence and often virulence (15, 18, 22, 77). Biofilms resist environmental stresses and adverse conditions better than free-living cells, have increased nutrient availability and can better avoid immune responses (5). A common feature of biofilms is that microorganisms are embedded in an extracellular matrix comprised mostly of EPS (14, 83). EPS is important for the structural and functional integrity of biofilms and determines its physicochemical and biological properties and has a role in adhesion, protection and facilitates community interactions (82). EPS provides protection from a variety of environmental stresses such as UV radiation, pH shifts, osmotic shock, and desiccation.

The role of biofilms in the environmental persistence and transmission of certain pathogens is also well recognized. Like *V. cholerae* (2, 50, 85), *Salmonella enterica* Typhimurium has the ability to form a rugose EPS-producing phenotype which has increased biofilm forming ability and is proposed to have a role in increased persistence in the environment (4). *Salmonella enteritidis* biofilms resistant to cleaning fluids have been shown to persist for at least 4 weeks in domestic toilets after episodes of salmonellosis (10). The finding that *E. coli* and *Salmonella* biofilms can be found on sprouts may make their eradication with antimicrobial compounds difficult and therefore increasing their persistence, resulting in ingestion and infection (25). Alginate EPS production by *P. aeruginosa* protects these strains against chlorine and may contribute to survival of these bacteria in chlorinated water systems (34). The importance of biofilms is also highlighted in the process of horizontal gene transfer since some results suggest that DNA exchange may be increased in bacteria that are attached to a surface and in biofilms rather than between free-swimming planktonic cells (23). This has implications in the transfer of genes encoding functions such as antibiotic resistance or virulence and overall persistence.

Clinically, biofilm formation is known to be a key factor in the establishment and persistence of several difficult to treat infections. Cystic fibrosis is caused by certain *P. aeruginosa* strains which express copious amounts of EPS and form biofilms in the lung (19, 29, 33). The EPS of these *P. aeruginosa* strains makes them

recalcitrant to antimicrobial treatment. Interestingly, like the EPS of *V. cholerae* (2, 50), alginate EPS production by *P. aeruginosa* protects these strains against chlorine and may contribute to survival of these bacteria in chlorinated water systems (34). Another example of a biofilm-mediated infection is chronic ear infection (otitis media) (21). Peridontitis is another example of a biofilm-mediated disease that results from chronic inflammation of the tissue supporting the gums and can lead to tooth loss. The main microbe causing this disease is *Porphyromonas gingivalis* (45). The EPS matrix of biofilms has the potential to physically prevent access of certain antimicrobial agents into the biofilm by acting as an ion exchanger, thereby restricting diffusion of compounds from the external milieu into the biofilm (30, 51, 52). *Helicobacter pylori* produces a biofilm that appears to be important in enhancing resistance to host defense factors and antibiotics and in promoting growth under low pH conditions *in vivo* (72). Biofilm bacteria can be up to 1,000-fold more resistant to antibiotic treatment than the same organism grown planktonically (30). Clinical biofilm infections are marked by symptoms that typically recur even after repeated treatments with antibiotics. Moreover, biofilm infections are rarely resolved by the host's immune system (16). Bacterial biofilms on prosthetic valves are the leading cause of endocarditis in patients who have undergone heart valve replacement. Among patients who develop these infections, the mortality rate is as high as 70% (42). Millions of catheters (e.g., central line, intravenous, and urinary catheters) are inserted into patients every year, and these implants serve as a potential surface for biofilms. Overall, it is thought that upwards of 60% of all nosocomial infections are due to biofilms. These biofilm-based infections can increase hospital stays by up to 2-3 days and cost upwards of $1 billion per year in added costs (6, 7).Unfortunately, the mechanisms leading to EPS production and the development of biofilms are not well understood and additional studies are needed to better understand the role of biofilms in pathogenesis and the signal factors promoting biofilm formation.

Preliminary Data

1. High frequency switching from EPSoff to EPSon in *V. cholerae*

Researchers studying the rugose phenotype of *V. cholerae* (and other species) have been impeded by the very

TABLE 3. Frequency of switching to rugose EPS production (HFRP) by *V. cholerae* strains.

Strains[a]	Serogroup/ Biotype	Source[b]	% Rugose colonies			
			Flask		Tube	
			30°C	37°C	30°C	37°C
N16961	O1/El Tor	C (1971)	24-38	42-51	68-74	60-80
C6709	O1/ElTor	C (1991)	1	23	15	70
NCTC 6585	O1/classical	C (1943)	33-48	44-45	0	0
AMS20A73	O1/classical	C (1945)	3	4	0	0
Aldova	O37	C (1965)	0	1	71-72	23-50
1803	non-O1	C (1992)	0	0	16	
87						
1837	O139	C (1992)	0	0.2	0	0-2
P44	non-O1	E (2000)	12	0	0	2
1085-93	O37	E (1993)	0	0	0.1	0
141-94	O70	E (1994)	0	0	0.3	0
928-93	O6	E (1993)	0	0.2	0.4	0

[a] Listed only are strains showing HFRP or spontaneous rugose colonies.
[b] C, clinical; E, environmental; Year isolated is in parentheses.

low (<1%) frequency of switching between smooth (EPSoff) and rugose cells (EPSon) *in vitro* (50, 75, 80, 81, 85). We have identified culture media and conditions, APW#3 (1% proteose peptone #3, 1% NaCl, pH 8.5.), which results in a high frequency shift of smooth cells (EPSoff) to the rugose phenotype (EPSon). We call this process high frequency rugose production (HFRP) (Table 3 and Fig. 2) (2).

We found that switching to the rugose phenotype at high frequency was more common in epidemic strains than in nonpathogenic strains. We found that 6/19 toxigenic isolates (32%) that were temporally and geographically unrelated and only 1/16 unrelated nontoxigenic strains (6%) could shift to the rugose phenotype (EPSon) and showed HFRP (T test; $P<0.05$) (Table 3). Of all the strains tested, El Tor strain N16961 had the highest switching rates (up to 80%). Reversion, albeit at a lower frequency, from the rugose phenotype to the smooth phenotype was also found showing that phenotypic switching is conditionally transient. These features suggest the switching process might be associated with phase variation-like mechanism. While not all epidemic strains could switch at high frequency, our results showing that switching at high frequency is more correlated with toxigenic strains suggests it is important in *V. cholerae* and suggest a link between this process and virulence. Consistent with previous studies (50), we found a low frequency (<0.5%) shift to the rugose phenotype in several strains. While it is possible that nonpathogenic strains might have to be grown under different conditions to stimulate switching to the EPSon rugose phenotype, this would still nevertheless indicate that there is a difference between clinical and nonpathogenic strains. We found that a sixth pandemic (classical biotype) strain, NCTC 6585, switched at high frequency to the rugose phenotype (up to 48%). HFRP was defined as a >3% shift from the smooth to rugose phenotype (2). To confirm the rugose variant of NCTC 6585 expressed rEPS, we performed transmission electron microscopy (TEM) on ruthenium red stained thin sections. For TEM, 2 day old smooth and rugose colonies on LB agar were removed as 0.5-cm^2 blocks then fixed and stained in a solution of 2% glutaraldehyde, 0.075% ruthenium red, 50 mM lysine monohydrochloride in 0.1 M cacodylate buffer (pH 7.2) for 1 h at room temperature then 18 h at 4°C. Samples were washed twice in 0.1 M cacodylate buffer (pH 7.2), encased in 2% molten Noble agar and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) overnight at 4°C. Samples were then dehydrated in 30-, 50-, 70- and 90% EtOH for 10 min each and twice in 100% EtOH for 15 min each, followed by two treatments with propylene oxide 15 min each then infiltrated using a 1:1 solution of propylene oxide and epon for 2 h at room temperature then in 3:1 epon/propylene oxide overnight. Samples were then placed in pure epon for 1 h, embedded in epon and put in a 60°C oven for 2 days then thin sectioned (50-80 nm thick). Sections were stained with uranyl acetate for 20 min then lead citrate for 20 min. Samples were examined under a JEOL 1200 EX II transmission microscope at 80 kV. TEM of rugose NCTC 6585 showed the presence of extracellular polysaccharide between cells and the absence of this material from smooth cells (Fig. 3). Using rugose-inducing it appears that all major epidemic clones of *V. cholerae* (classical, El Tor and O139) can shift to the rugose phenotype.

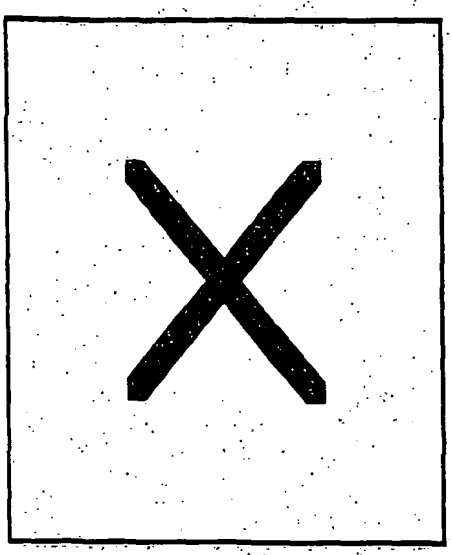
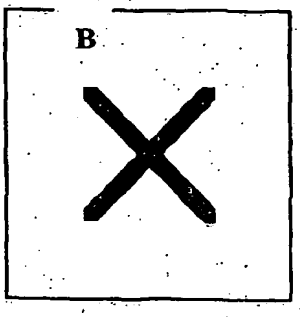
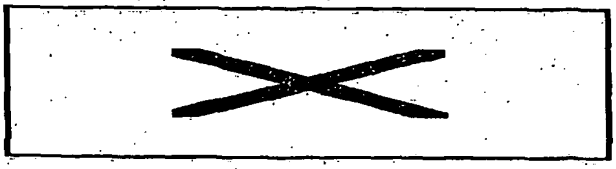


Fig. 2. Colony morphology of smooth and rugose variants of *V. cholerae*. Panel A, colonies at 24 h; Panel B, colonies at 72 h.



promotes high-level resistance



2. The rugose phenotype chlorine

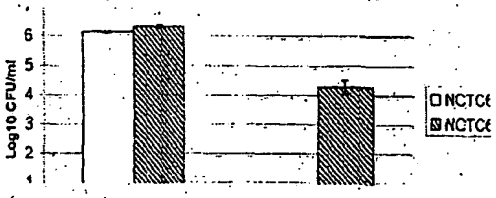


Fig. 4. Effect of chlorine on the su

Production of rEPS is known to promote resistance of El Tor strains to a variety of environmental stresses such as chlorine, UV light, hydrogen peroxide, and complement-mediated bactericidal activity (50, 63, 78, 85). In order to determine whether rugose cells of 6th pandemic classical biotype strain NCTC 6585 promoted resistance to environmental stresses, we exposed smooth and rugose variants to chlorine. Chlorine resistance was assayed (four independent experiments) by using a 1:50 dilution of an overnight culture of NCTC 6585 in 3 ml of fresh LB (Miller) broth. Cultures were then incubated statically at 37°C for 3 h until CFU/ml ~2 x 10^8 CFU/ml, the cells harvested by centrifugation and resuspended in phosphate buffered saline (PBS) (pH 7.2) containing 3 mg/L free chlorine (sodium hypochlorite, Sigma). Following 5 min exposure to 3 mg/L chlorine, cultures were serially diluted and plated on LB agar to determine the number of surviving cells. Consistent with El Tor strain 92A1552 (85), rugose NCTC 6585 cells were 10,000-fold more resistant to chlorine (5 min. exposure to 3 mg/L) than smooth cells (Fig. 4). These findings are the first to report the rugose phenotype by classical biotype strains and shows that rEPS also promotes the survival of classical biotype strains.

3. Switching to EPSon and the rugose phenotype promotes biofilm formation

As the rugose phenotype can promote biofilm formation in El Tor and O139 strains, we tested the ability of smooth and rugose variants of N16961 (El Tor), NCTC6585 (classical) and Aldova (non-O1/non-O139) strains to form biofilms using previously described methods (79). Glass test tubes containing 500 µl LB broth were inoculated with a 1:100 dilution of overnight culture of each variant.

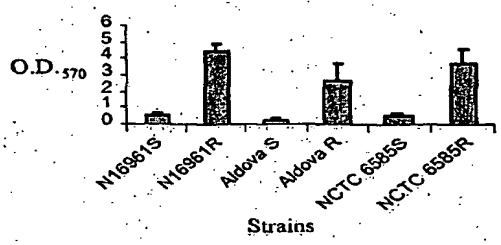


Fig. 5. Biofilm formation by smooth and rugose colony variants of *V. cholerae*.

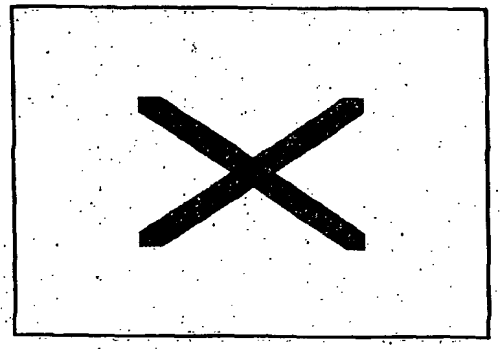


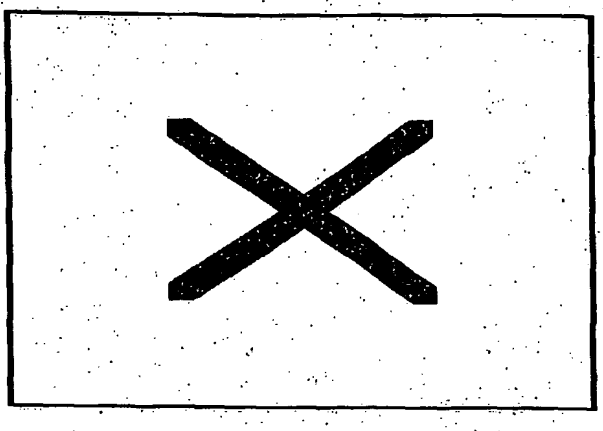
Fig. 6. Biofilm formation in *V. cholerae*. Panel A, NCTC 6585 smooth (no biofilm); panel B, N16961 smooth cels (no biofilm); panel C, NCTC 6585 rugose (biofilm); panel D, N16961 rugose (biofilm).

These cultures were then incubated statically at room temperature for 24 h. Culture supernatants were then discarded, tubes rinsed vigorously with distilled water to remove non-adherent cells, filled with 600 µl 0.1% crystal violet (Sigma), incubated for 30 min at room temperature and again rinsed with water. Quantitative biofilm formation was assayed by measuring optical density at 570 nm of the solution produced by extracting cell associated dye with 600 µl DMSO (Sigma). Consistent with other studies (85), the results show that rugose variants of all strains tested had significantly greater (~7-fold) biofilm forming ability than smooth cells (Fig. 5 and 6) and that EPS is essential for *V. cholerae* biofilm formation.

4. *V. cholerae* can switch to the rugose phenotype (EPSon) in the environment

The supposition that switching to EPSon and the rugose phenotype promotes the survival of *V. cholerae* in the environment is based on the premise that switching to EPSon occurs in the environment. However, there have been no reports detecting rugose *V. cholerae* from environmental (or clinical) sources. Unfortunately, there is no current enrichment method for isolating rugose strains from the environment and while TCBS is a selective and differential media for *V. cholerae*, we have found that TCBS inhibits (masks) the rugose phenotype (2).

To test whether smooth cells switch to the rugose phenotype in natural environmental water samples, we used natural lake water from Lake Kittamaqundi that is located on the edge of the City of Columbia, Howard County, Maryland. Lake Kittamaqundi is a 27-acre man-made lake approximately 1 mile long by 1/8th mile wide and has a maximum depth of 7 feet. The Chesapeake Bay, which is approximately only several miles away, is known to be a natural reservoir of *V. cholerae*. We collected fresh water samples from the lake between the warmer months of March-September, 2002. At collection the lake water had pH 7.6 and Na^+ and Cl^- concentrations of 6 mM and 2 mM, respectively. Lake water was autoclaved for 1 h prior to use. In this study, *V. cholerae* strain N16961 was grown in LB broth overnight at 37°C, centrifuged, washed twice with 0.85% NaCl, resuspended in PBS, appropriately diluted and inoculated into 100 ml lake water to a final concentration of 10^4-10^5 cfu/ml confirmed by plate count. Microcosms were incubated statically at room temperature in the dark and at appropriate time intervals aliquots were plated onto LB agar and plate counts and colony morphology were determined. The preliminary results after approx. 6 months sampling suggests that *V. cholerae* N16961 can persist under these conditions with only 1-log decrease in viability. Importantly, N16961 was able to switch from the smooth (EPSoff) to rugose (EPSon) phenotype at high frequency (up to 16%) under these conditions by day 50 (Fig. 7). An advantage of this study is that it closely mimics a true environmental scenario and the switching of a wildtype strain of N16961. While these studies could be extended, these results suggest that *V. cholerae* can shift to the rugose phenotype in natural environments.



5. Compositional analysis of the rEPS in *V. cholerae*

Our previous findings were the first to report the rugose phenotype in a classical (6th pandemic) biotype strain of *V. cholerae*. In order to determine the structural composition of the rugose variant of classical biotype strain NCTC 6585 and to compare it to polysaccharides in other strains, a rugose colony of classical biotype strain NCTC 6585 was inoculated into APW#3 and incubated at 37°C for 3 days under static conditions to promote EPS production and biofilm formation. To harvest the EPS, the cultures were filtered using a large (10 µm) pore size filter (VWR). The biofilm was washed once gently with PBS to remove planktonic cells, transferred to a fresh tube and 3 mm glass beads added to disrupt the biofilm. The sample was then centrifuged at 20,000 rpm (50,000 x g) for 16 h at 4°C to remove cell debris and other contaminants. The supernatant was passed though a Detoxi-Gel Affinitypak column (Polymixin B immobilized on agarose column) (Pierce) to remove any traces of LPS from the sample and DNase and RNase (final conc. 100 µg/ml) was added then incubated at 37°C for 4 h. Proteinase K (final conc. 100 µg/ml) was added and incubated at 37°C overnight followed by 60°C for 15 min. Following the addition of 3 volumes of 95% ethanol, the mixture was precipitated overnight at 4°C then centrifuged at 12,000 rpm for 20 min. The precipitated EPS was washed twice, first with 80% ethanol and then with 95% ethanol. The EPS precipitate was resuspended in 0.5 ml MQ, incubated at −80°C for 2 h, lyophilized for 4 h, then analyzed by combined gas chromatography/mass spectrometry (GC/MS) and performed by the Complex Carbohydrate Research Center in Atlanta, Georgia.

The analysis in Table 4 showed that the rugose EPS (rEPS) for 6th pandemic strain NCTC 6585 differs markedly from the EPS of 7th pandemic strain 92A1552 which has glucose as the predominant sugar (85) and strain TSI-4 which has mannose as the predominant sugar (75). The compositional analysis result also suggests that the extracellular carbohydrate described here is quite different from O1 LPS which typically contains large amounts of perosamine and quinovosamine (62). In contrast to the results of El Tor strain 92A1552 in which 4-linked galactose and 4-linked glucose were the dominant linkages (85), the glycosyl linkage analysis using gas chromatography-mass spectrometry (GC-MS) performed on the classical biotype strain show that the predominant linkage is a 4-linked galactosyl residue and may represent the backbone of the saccharide. Our detailed structural analysis results provide compelling evidence that there are differences in structure of the rEPS and the rugose phenotype between *V. cholerae* strains which can be further studied.

TABLE 4. Glycosyl composition and linkage analysis of rEPS from strain NCTC 6585

Sugar	Glycosyl Composition	Glycosyl linkage		
	%	Glycosyl residue[a]	%	
Rhamnose	8.92	terminal linked –fucosyl residue		9.8
Fucose	10.46	terminal linked –glucosyl residue	7.9	
Mannose	4.68	3 linked –glucosyl residue	8.8	
Galactose	18.71	2 linked –glucosyl residue	15.0	
Glucose	9.29	4 linked –manosyl residue	14.2	
GalNAc	16.86	4 linked –galactosyl residue	24.8	
GlcNAc	27.65	2,3,4 linked –fucosyl residue	7.7	
		2,3 linked –manosyl residue	11.8	

[a] All residues are in the pyranose (p) form.

6. Molecular basis of high frequency switching from EPSoff (smooth) to EPSon (rugose)

Our identification of conditions that promote the switch to the rugose phenotype at high frequency was exploited in the development of a screening assay to identify genes involved in the molecular switch from the smooth to the rugose phenotype. To identify the genes involved in the molecular switch, mini-Tn*5km2* mutagenesis was used (20, 39). Tn5 is contained on the R6K-based plasmid pUT/mini-Tn5 *Km2* (or pUTKm) that is derived from suicide vector pGP704 (48) and can only be maintained in donor strains (e.g. a λ*pir* lysogen of *E. coli*) that produce the R6K-specified λ*pir* protein which is an essential replication protein for R6K and plasmids derived therefrom. It also carries the origin of transfer, *oriT*, of plasmid RP4 which enables efficient conjugal transfer. Delivery of the donor plasmid pUTKm into recipient cells is mediated by the cognate transposase encoded on the plasmid at a site external to the transposon. An advantage of this mutagenesis system is the stability of the Tn5 insertion since the cognate transposase is not carried with the transposon during transposition. Thus, each mutant has only a single Tn5 insertion to screen. In these studies, we mated *E. coli* S17 λpir (pUT/mini-Tn5 Km) with a smooth N16961 (EPSoff) strain. We obtained 14,500 Tn5 mutants from at least 10 independent conjugations and have identified 43 mutants operationally defined as HFRP-negative. These mutants do not produce any detectable rugose colonies under rugose-inducing conditions. To identify the transposon insertion site in these mutants, we used a non-laborious arbitrary primed PCR method followed by DNA sequencing similar to that described previously (9). Briefly, arbitrary PCR was performed in two steps: in the first reaction, chromosomal DNA of the mutant was used as a template for PCR using primers reading out from both sides of the transposon and two arbitrary primers. These primary reactions yielded numerous amplicons including some that were derived from the junction of the transposon insertions. The products of the first-round PCR were purified by Geneclean and amplified using a second pair of outward transposon primers external to the first pair and an arbitrary primer corresponding to the constant region of the original arbitrary primers. This secondary PCR reaction serves specifically to amplify products of the first PCR that include transposon junctions. Amplified fragment ranged between 100- to 800-bp. The products that gave the strongest bands were from agarose gels and sequenced using the same transposon and arbitrary primers used in the second-round PCR. Sequencing was performed using an automated DNA sequencer (model 373A, Applied Biosystems) using the Prism ready reaction dye deoxy termination kit (Applied Biosystems) according to the manufacturer's instructions. We have successfully sequenced and identified the transposon insertion site in the 43 mutants and have performed a BLAST search against the published *V. cholerae* N16961 genome to identify the disrupted genes (36). A summary of these results are shown in Table 5.

TABLE 5. Representative HFRP mutants of *V. cholerae* N16961

Mutant[a]	Locus[b]	Predicted protein	Predicted function
DK568 (2)	VC0243	RfbD	LPS biosynthesis, GDP-mannose 4,6 dehydratase

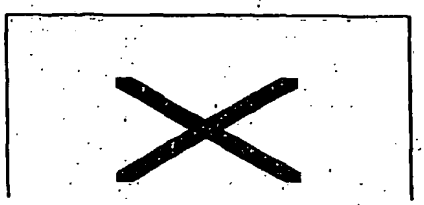
DK623 (1)	VC0244	RfbE	LPS biosynthesis, perosamine synthase
DK578 (2)	VCA0744	GalE	LPS biosynthesis, UDP-glucose 4-epimerase
DK589 (1)	VC0920	Vps (EpsF)	EPS biosynthesis, glycosyl transferase
DK576 (2)	VC0921	Vps (Wzx)	EPS, polysaccharide export
DK588 (7)	VC0922	Vps	EPS, hypothetical protein
DK562 (13)	VC0665	VpsR	EPS biosynthesis, σ^{54} transcriptional activator
DK614 (10)	VC2628	AroB	aromatic a.a. synthesis, 3-dehydroquinate synthase
DK625 (1)	VC2629	AroK	aromatic a.a. synthesis, shikimate kinase
DK630 (1)	VC0344	AmiB	N-acetylmuramoyl-L-alanine amidase
DK567 (3)	VC0653	RocS	regulatory, contains GGDEF and EAL domains

[a] Numbers in brackets indicate number of mini-Tn5 mutants having insertions in same locus.
[b] Loci and predicted proteins derived from the *V. cholerae* N16961 TIGR sequencing project.

Previous transposon mutagenesis studies (including one by our group) have identified gene mutations that result in stable rugose-to-smooth mutants (1, 78, 85). In contrast, taking advantage of our conditions that promote switching to rugose phenotype, we performed transposon mutagenesis on a smooth strain and screened for stable mutants that were unable to switch to the rugose phenotype under rugose-inducing conditions. While our findings revealed mutants with defects in genes previously identified with roles in the rugose phenotype such as several rEPS biosynthesis (*vps* operon) and rEPS regulatory genes (*vpsR)* and LPS genes *(galE)*, our screen identified mutants sustaining insertions in previously unidentified genes. These newly identified mutants could be clustered into several functional groups coding for LPS (*rfbD* and *rfbE*) whereby impairment in the LPS structure might promote shutdown of the rugose (EPSon) phenotype; genes involved in aromatic amino acid synthesis (*aroB* and *aroK*) whereby aromatic amino acid synthesis genes might be directly or indirectly associated with the rugose phenotype; a gene involved in cell wall hydrolysis (*amiB*) and a novel locus, VC0653, designated "*pdeA*-like" in the N16961 genomic database, which we have now termed RocS (for regulation of cell signaling) encoding a putative protein containing GGDEF and EAL domains.

It is important to note that we isolated three independent mutants containing mutations in *rocS* (VC0653) from three independent conjugations. These results suggest that RocS has an important role in rEPS production, the rugose phenotype and in biofilm formation. The defect in the rugose phenotype in the *rocS* mutant was not explained by differences in growth rate between the wildtype (N16961) and RocS (DK567) cells as determine using a spectrophotometer (data not shown). The presence of GGDEF domains in all proteins known to be involved in the regulation of cellulose (β-1,4-glucan) synthesis (8) Cellulose production in *Acetobacter xylinum*, *Rhizobium leguminosarum* bv. *trifolii* and *Agrobacterium tumefaciens* is modulated by the opposing effects of two enzymes, diguanylate cyclase (Dgc) and c-di-GMP phosphodiesterase (PdeA), each controlling the level of c-di-GMP in the cell (Fig. 8) (3, 46, 66, 67). Diguanylate cyclase acts as a positive regulator by catalyzing the formation of c-di-GMP which specifically activates cellulose production while the phosphodiesterase cleaves c-di-GMP and negatively regulates cellulose. c-di-GMP is predicted to be a reversible, allosteric activator (effector) of cellulose biosynthesis (66). Furthermore, genetic complementation studies using genes from different species encoding proteins with GGDEF domains as the only element in common suggest that the GGDEF domain has a role in diguanylate cyclase activity and is important in modulating the level of c-di-GMP (8). We have discovered that this protein homologue regulates EPS production, biofilm formation, motility and presumably other phenotypes.

A BLAST search of the *V. cholerae* RocS shows that it is highly conserved and has significant homologues in a wide variety of other species including *P. aeruginosa* (PA0575; 42% id; $5e^{-92}$), *B. anthracis* (BA5593; 37% id; $6e^{-90}$), *Ralstonia solanacearum* (RSc0588; 36% id; $4e^{-88}$) and *Acetobacter xylinum* (c-di-GMP diguanylate cyclase Dgc; 40%, $9e^{-82}$). Although *dgc* and *pdeA* genes share some homology and have similar domain architecture, our finding that the *V. cholerae* RocS mutant is unable to produce an EPS is more consistent with a *dgc* mutant and RocS has higher similarity with *A. xylinum* Dgc compared to PdeA (data not shown). Recent reports have identified "RocS' homologs in *P. aeruginosa* that appear essential for biofilm formation (13) and in *V. parahaemolyticus* that regulate capsular polysaccharide production (35). Additionally, the autoaggregation phenotype (which is typical of the rugose phenotype) in the plague bacterium *Yersinia pestis* requires the GGDEF-containing protein HmsT (43). Although homologous regulatory GGDEF-containing proteins have been found in several species and have been associated with wrinkled colonies, EPS production or biofilm formation, their role in regulating these processes has not been able to be well studied, in part due to the lack of available reagents. Since there is evidence suggesting that GGDEF-containing proteins possess nucleotide cyclase activity (8,



58, 68, 73) and are widespread in bacteria (17, 28), we propose that modulation of c-di-GMP and its analogs, either directly or indirectly can be used in various applications to regulate prokaryotic and eukaryotic phenotypes.

The bacterial cell wall is typically composed of a heteropolymer known as murein or peptidoglycan. Many Gram-negative bacteria degrade up to 50% of their murein per generation and recycle it to form new murein (31, 32, 55). N-acetylmuramoyl-L-alanine amidases are often associated with autolysis or microbial cell wall hydrolysis. Surprisingly, enzymes in Gram-negatives that cleave the septum such as AmiB have only recently been studied in a few species and in *E. coli*, AmiB mutants are found growing as long chains of unseparated cells (37, 40). In *Azotobacter vinelandii*, an N-acetylmuramoyl-L-alanine amidases is linked to alginate production by the ability of *A. vinelandii* cells to recycle their cell wall (53). Our identification that the AmiB (N-acetylmuramoyl-L-alanine amidase) was linked to switch to the rugose phenotype prompted us to further study the VC0344 locus and its predicted protein.

A BLAST search shows that the *V. cholerae* AmiB sequence has high similarity to N-acetylmuramoyl-L-alanine amidases found in a wide variety of species including *P. aeruginosa* ($7e^{-78}$), *S.* enterica Typhi ($7e^{-69}$), *E. coli* O157:H7 ($6e^{-57}$) and *Y. pestis* ($6e^{-50}$). AmiB in *V. cholerae* is predicted to be a 59-kDa protein that is unusually rich in serine (9.5%), proline (6%) and threonine (6%). Such a composition is common in protein domains associated with the cell wall in Gram-positive bacteria (26) and is similar to a putative peptidoglycan hydrolase of *Lactococcus lactis* (*acmB*) (41). In *V. cholerae*, like *E. coli* and *Y. pestis*, an *amiB* amidase is located immediately upstream of *mutL* which has a role in DNA mismatch-repair (56, 74). A computer analysis using PSORT shows that *V. cholerae* AmiB is predicted to have a cleavable N-terminal signal sequence and an analysis using TMpred strongly predicts that AmiB has two transmembrane domains (score 2363), one at the N-terminal end (a.a. 10-29) which could also represent an N-terminal signal anchor sequence and another transmembrane domain at the C-terminal end (a.a. 446-465). The *V. cholerae* AmiB is predicted to contain a LysM (lysin motif) domain at its C-terminal end and this has been found in enzymes involved in cell wall degradation (11). Interestingly, the *V. cholerae* AmiB contains a Arg-Gly-Asp (RGD) motif that is often associated with a surface binding domain for various mammalian adhesion proteins.

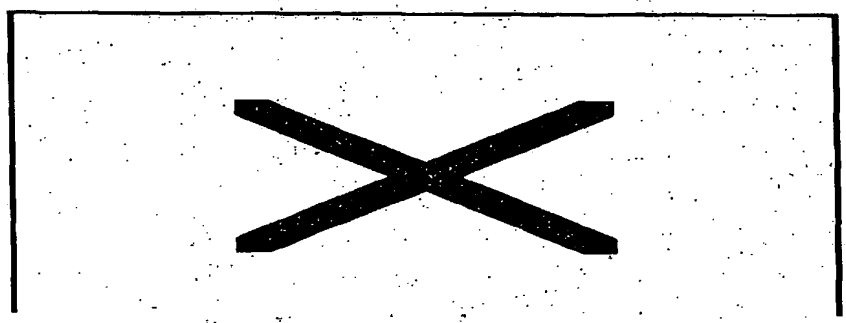


Fig. 9. Effect of an AmiB mutation on the cellular morphology of *V. cholerae* strain N16961. Panel A, wildtype; B, AmiB mutant. Note that the AmiB mutant cells have a difference in morphology and show an increase in overall cell size and the appearance of numerous cells in chains. Both images obtained using 1000x magnification.

Since AmiB has been associated with septation in other species such as E. coli (37, 38), we determined whether the *V. cholerae* AmiB mutant was affected in its cellular morphology as well as the rugose phenotype. In our studies, a single 18 h colony on an LB plate from wildtype N16961 and AmiB mutant DK630 was resuspended in 1 ml PBS and a 50 µl aliquot smeared onto a glass slide, heat fixed then stained with 0.1% crystal violet for 30 sec. The slide was then rinsed with dH_2O, dried and cell morphology observed using a Zeiss Axioskop epifluorescence microscope (Carl Zeiss, Inc. NY). The images were acquired using an AxioCam Mrm camera (Carl Zeiss, Inc. NY). Examination of the cells showed an obvious difference between the AmiB mutant and the wildtype strain in the morphology and arrangement of the cells (Fig. 9). Many cells of the AmiB mutant were altered in shape and some were dramatically increased in cell size (length and width). The AmiB mutant appeared to have a higher percentage of cells in chains. This finding suggests that cell division and septation might be affected. We found no difference in growth rate between the wildtype (N16961) and AmiB mutant (DK630) as determined using a spectrophotometer (data not shown) suggesting that the apparent difference in cell structure is not due to differences in growth rate. While the findings of cells grown on LB plates bred true following subculture, we did not find obvious dramatic differences between the strains when grown in LB broth (data not shown). Although further studies are required to analyze the cellular structure of the AmiB mutant in more detail, such as using electron microscopy, the results of our studies presented here suggest there is a link between cell division, structure or septation and the rugose phenotype of *V. cholerae*. Our findings provide evidence for a new function for a prokaryotic amidase, namely its importance in the switch to the rugose phenotype and biofilm formation.

The importance in regulating *vps* biosynthetic genes in *V. cholerae* led us to further study several *vpsR* transposon mutants, designated DK562 and DK581, respectively. VpsR is encoded by the locus VC0665 and is a 444 amino acid

protein with high similarity to the family of σ54 response regulators such as NtrC, AlgB, and HydG (1, 84). We found that supplying plasmid pDK104 containing *vpsR* on a 2.61-kb PCR fragment which was obtained from strain N16961 using PCR primers KAR486 (5'-CGGGATCCCGCTAAGTCAGAGTTTTTATCGC-3') and KAR487 (5'-TCCCCGCGGGTCGGTGGTTTTGATCGTGT-3'), digested with *Bam*HI and *Sac*II, respectively, and suitably cloned into the low copy vector pWSK29 (76), can restore switching to the rugose phenotype in both these *vpsR* mutants. These findings confirm that the defect in switching to the rugose phenotype in these mutants is due to the mutation in *vpsR*.

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Methods and Uses of Modlating Microbial Cyclic Dinucleotides

A useful method to regulate the level of biofilm formation, extracellular polysaccharide, virulence and growth of microbial species by modulating the level of c-di-GMP (or analogs)

Various embodiments of the invention are disclosed in the manuscripts attached hereto entitled "Methods and Uses of Modulating Microbial Cyclic Dinucleotides" and "Identification of Genes Involved intheSwitch Between Smooth and Rugose Phenotypes of *Vibrio Cholerae*". Also incorporated by reference is a disclosure form, and background information related to Cholera and Biofilms.

Identification of Genes Involved in the Switch Between the Smooth and Rugose Phenotypes of *Vibrio cholerae*

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Running title: Rugose *V. cholerae*

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Cholera is a diarrheal disease of humans that results in significant morbidity and mortality [1,2]. Cholera affects more than 75 countries and every continent (Communicable Disease Surveillance and Response, World Health Organization; www.who.org). Cholera is acquired by drinking fecally contaminated food or water containing pathogenic *Vibrio cholerae* that can colonize the small intestine and release cholera toxin (CT) resulting in massive secretory diarrhea and death if untreated [2]. Because of its high death-to-case ratio, persistence in water supplies and its ability to occur in explosive epidemic form, cholera is a public health concern.

V. cholerae can alter its phenotype and reversibly switch from an EPSoff (smooth colony morphology) to an EPSon (rugose colony morphology) in which the cells are embedded in an extracellular polysaccharide or rugose exopolysaccharide (rEPS) and demonstrate a wrinkled colony morphology [3,4]. The switch to EPSon and the rugose phenotype promotes biofilm formation [5-7]. The rugose variant is highly chlorine resistant and shows increased resistance to killing by acid, UV light and complement-mediated serum bactericidal activity [5,6,8]. Therefore, switching to EPSon and the rugose phenotype might be important in promoting survival in particular environments. Rugose strains are virulent and cause fluid accumulation in rabbit ileal loops, produce diarrhea in human volunteers and are highly resistant to complement-mediated bactericidal activity [5,6,8]. Research by our group and others has also shown that production of *V. cholerae* EPS is linked to the type II general extracellular protein secretion pathway which is also involved in secretion of important virulence factors [9,10].

The *vps* (*Vibrio* polysaccharide) gene cluster in *V. cholerae* carries the structural genes for the biosynthesis of rEPS [8]. The *vps* gene cluster is thought to be comprised of two closely located but separate operons in which *vpsA* and *vpsL* represent the first genes of each opcron [8,11]. Transcription of *vpsA* and *vpsL* is regulated by VpsR (a homolog of σ54 transcriptional activators) by a mechanism that is not well understood [11]. VpsR has high homology to NtrC, AlgB and HydG bacterial enhancer-binding protein that activates transcription after phosphorylation of its receiver domain by an associated sensor kinase protein [12]. Although previous studies have found that a mutation in HapR in *V. cholerae* strain 3083 is linked to the rugose phenotype by some unknwon mechanism [13], CytR was shown to repress rEPS transcription of *vps* genes and the associated biofilm formation [14] and we found also that switching to the rugose phenotype was independent of ToxT, LuxS and RpoS [15], the molecular basis underlying switch from the smooth to the rugose phenotype of *V. cholerae* is still not well understood.

Early studies on the rugose phenotype of *V. cholerae* were impeded by the very low frequency of switching to EPSon and the rugose phenotype under the conditions tested [6,8,16]. We recently identified conditions that promote the rapid shift (up to ~80%) to the rugose phenotype in a process we called high frequency rugose production (HFRP) [15]. We found there are differences in the expression and stability of the phenotype between epidemic strains and that the ability to switch at high frequency was more common in epidemic *V. cholerae* strains than in nonpathogenic strains [15]. This suggests that the ability to switch to the rugose phenotype is important in *V. cholerae* and might provide an adaptive advantage under specific

conditions. In pursuit of our interests in the factors involved in the emergence and pathogenesis of *V. cholerae* and the rugose phenotype, we exploited our recent finding of rugose-inducing conditions to study the genes involved in the switch between the smooth (EPSoff) and rugose (EPSon) phenotypes of *V. cholerae*.

Genetic screen for genes with roles in the switch to the rugose phenotype. Our identification of culture conditions that promote the switch to the rugose phenotype at high frequency (HFRP) [15] was exploited in the development of an assay to identify genes involved in the molecular switch from the smooth to the rugose phenotype. In our previous studies, we reported that incubation of cells in a medium we called APW#3 resulted in a high frequency of smooth N16961 cells (up to ~80%) switching to the rugose phenotype [15] (Fig. 1). To identify the genes involved in the molecular switch from the smooth (EPSoff) to the rugose (EPSon) phenotype, mini-Tn5*km2* mutagenesis was used [17,18]. Tn5 is contained on the R6K-based plasmid pUT/mini-Tn5 Kan (or pUTKm) that is derived from suicide vector pGP704 [19] and can only be maintained in donor strains (e.g. a λ*pir* lysogen of *Escherichia coli*) that produce the R6K-specified λ*pir* protein which is an essential replication protein for R6K and plasmids derived therefrom. It also carries the origin of transfer, *oriT*, of plasmid RP4 which enables efficient conjugal transfer. Delivery of the donor plasmid pUTKm into recipient cells is mediated by the cognate transposase encoded on the plasmid at a site external to the transposon. An advantage of this mutagenesis system is the stability of the Tn5 insertion since the cognate transposase is not carried with the transposon during transposition. Thus, each mutant has only a single Tn5 insertion to screen. In our studies, we mated *E. coli*

S17 λpir (pUT/mini-Tn5 Km) with smooth N16961 (EPSoff) cells and obtained 14,500 mini-Tn5 mutants from 30 independent conjugations and were subsequently stored in wells of microtiter plates. We performed a high throughput screen for HFRP mutants in which the transposon mutants were inoculated into 200 µl APW#3 media in wells of microtiter plates, incubated for 48 h then replica plated onto LB agar and incubated for 24-48 h after which the colony morphology was visually examined. Using this approach, we identified 43 mutants operationally defined as HFRP-negative that did not produce any rugose colonies. We further confirmed that these mutants were stable and defective in switching to the rugose phenotype under HFRP-inducing conditions by inoculating a colony into 3 ml APW#3 in glass test tubes and incubating statically for 48 h at 37°C. Sterile glass beads (4 mm diameter) were then added and the cultures vortexed to disrupt any aggregates of rugose cells. Appropriate dilutions of each culture were plated on LB agar and colonies were counted by standard plate count to determine the total CFU/ml and the frequency of rugose cells. The 43 mutants identified and tested by these screening methods did not produce any detectable rugose colonies under rugose-inducing (HFRP) conditions and were further studied.

To identify the transposon insertion site in these mutants, we used a non-laborious arbitrary primed PCR method followed by DNA sequencing similar to that described previously [20]. Briefly, arbitrary PCR was performed in two steps: in the first reaction, chromosomal DNA of the mutant was used as a template for PCR using primers reading out from both sides of the transposon and two arbitrary primers. These primary reactions yielded numerous amplicons including some that were derived from the junction of the transposon

insertions. The products of the first-round PCR were purified by Geneclean and amplified using a second pair of outward transposon primers external to the first pair and an arbitrary primer corresponding to the constant region of the original arbitrary primers. This secondary PCR reaction serves specifically to amplify products of the first PCR that include transposon junctions. Amplified fragment ranged between 100- to 800-bp. The products that gave the strongest bands were from agarose gels and sequenced using the same transposon and arbitrary primers used in the second-round PCR. Sequencing was performed using an automated DNA sequencer (model 373A, Applied Biosystems) using the Prism ready reaction dye deoxy termination kit (Applied Biosystems) according to the manufacturer's instructions. We have successfully sequenced and identified the transposon insertion site in the 43 mutants and have performed a BLAST search against the published *V. cholerae* N16961 genome to identify the disrupted genes [21]. A summary of these results are shown in Table 1.

Previous transposon mutagenesis studies (including one by our group) have identified gene mutations that result in stable rugose-to-smooth mutants [7,8,22]. In contrast, taking advantage of our conditions that promote switching to rugose phenotype, we performed transposon mutagenesis on a smooth strain of N16961 and screened for stable mutants that were unable to switch to the rugose phenotype under rugose-inducing conditions. While our findings revealed mutants with defects in genes previously identified with roles in the rugose phenotype such as several biosynthesis (*vps* operon) and regulatory genes (*vpsR)* and LPS genes *(galE)*, our screen identified mutants sustaining insertions in previously unidentified genes. These newly identified mutants could be clustered into several functional groups coding for LPS (*rfbD* and *rfbE*) whereby impairment in the LPS structure might promote the shutdown of the rugose (EPSon)

phenotype; genes involved in aromatic amino acid synthesis (*aroB* and *aroK*) whereby aromatic amino acid synthesis genes might be directly or indirectly associated with the rugose phenotype; a gene involved in cell wall hydrolysis (*amiB*) and a novel locus, VC0653, designated "*pdeA*-like" in the N16961 genomic database, which we have now termed RocS (for regulation of cell signaling) encoding a putative protein containing GGDEF and EAL domains.

VpsR has an essential role in switching to the rugose phenotype. The importance in regulating *vps* biosynthetic genes in *V. cholerae* led us to further study several *vpsR* transposon mutants, designated DK562 and DK581. VpsR, encoded by the locus VC0665 is a predicted 444 amino acid protein with high similarity to the family of σ54 response regulators such as NtrC, AlgB, and HydG [11,22]. We found that supplying plasmid pDK104 containing *vpsR* on a 2.61-kb PCR fragment which was obtained from strain N16961 using PCR primers KAR486 (5'-CGGGATCCCGCTAAGTCAGAGTTTTTATCGC-3') and KAR487 (5'-TCCCCGCGGGTCGGTGGTTTTGATCGTGT-3'), digested with *Bam*HI and *Sac*II, respectively, and suitably cloned into the low copy vector pWSK29 [23], can restore switching to the rugose phenotype in both these *vpsR* mutants. These findings confirm that the defect in switching to the rugose phenotype in these mutants is due to the mutation in *vpsR*. Since VpsR is predicted to be a transcriptional activator we speculated whether it controlled motility in *V. cholerae*. Motility was determined in a swarm plate assay by measuring the swarm diameter of each zone after stabbing an equal amount of *V. cholerae* cells (grown in LB broth) into LB media containing 0.3% agar and incubation at 37°C for 4 h. The motility tests performed on the VpsR mutants (DK562 and DK581) showed that the mutants are

consistently ~50% reduced in its motility compared to the parent N16961 (data not shown). Since *V. cholerae* cells are typically motile and motility is important for virulence [24,25], it is tempting to speculate that VpsR might also have a role in virulence of *V. cholerae*. Although VpsR is important in regulating EPS (*vps*) biosynthesis genes and potentially other phenotypes, the conditions promoting VpsR expression and its mechanism of regulating *vps* genes is not well understood.

The AmiB amidase has a role in the switch to the rugose phenotype. The AmiB (N-acetylmuramoyl-L-alanine amidase) protein is encoded by the *amiB* gene (VC0344). Since we have previously found that rugose strains of *V. cholerae* are affected in motility [15], we tested whether *amiB* mutants are affected in their motility. Motility assays using soft (0.3%) agar showed that the AmiB mutant (strain DK630) was consistently >50% reduced in its motility (10 mm zone) compared to its parent N16961 (26 mm zone) (Fig. 2). These results suggest that AmiB affects motility and the rugose phenotype in *V. cholerae*.

The bacterial cell wall is typically composed of a heteropolymer known as murein or peptidoglycan. Many Gram-negative bacteria degrade up to 50% of their murein per generation and recycle it to form new murein [26-28]. N-acetylmuramoyl-L-alanine amidases are often associated with autolysis or microbial cell wall hydrolysis. Surprisingly, enzymes in Gram-negatives that cleave the septum such as AmiB have only recently been studied in a few species and in *E. coli*, AmiB mutants are found growing as long chains of unseparated cells [29,30]. In *Azotobacter vinelandii*, an N-acetylmuramoyl-L-alanine amidases is linked to alginate production by the ability of *A. vinelandii* cells to recycle their cell wall [31].

A BLAST search shows that the *V. cholerae* AmiB sequence has high similarity to N-acetylmuramoyl-L-alanine amidases found in a wide variety of species including *Pseudomonas aeruginosa* ($7e^{-78}$), *Salmonella enterica* Typhi ($7e^{-69}$), *E. coli* O157:H7 ($6e^{-57}$) and *Yersinia pestis* ($6e^{-50}$). AmiB in *V. cholerae* strain N16961 is predicted to be a 59-kDa protein that is unusually rich in serine (9.5%), proline (6%) and threonine (6%). Such a composition is common in protein domains associated with the cell wall in Gram-positive bacteria [32] and is similar to a putative peptidoglycan hydrolase of *Lactococcus lactis* (*acmB*) [33]. In *V. cholerae*, like *E. coli* and *Y. pestis*, *amiB* is located immediately upstream of *mutL* which has a role in DNA mismatch-repair [34,35]. A computer analysis using PSORT shows that *V. cholerae* AmiB is predicted to have a cleavable N-terminal signal sequence and an analysis using TMpred strongly predicts that AmiB has two transmembrane domains (score 2363), one at the N-terminal end (a.a. 10-29) which could also represent an N-terminal signal anchor sequence and another transmembrane domain at the C-terminal end (a.a. 446-465). The *V. cholerae* AmiB is predicted to contain a LysM (lysin motif) domain at its C-terminal end and this has been found in enzymes involved in cell wall degradation [36]. Interestingly, the *V. cholerae* AmiB contains a Arg-Gly-Asp (RGD) motif that is often associated with a surface binding domain for various mammalian adhesion proteins.

Since AmiB has been associated with septation in other species such as *E. coli* [29,37], we determined whether the *V. cholerae* AmiB mutant was affected in its cellular morphology as well as the rugose phenotype. In our studies, a single 18 h colony on a LB plate from the wildtype N16961 and AmiB mutant strain DK630 was resuspended in 1 ml PBS and a 50 µl aliquot

smeared onto a glass slide, heat fixed then stained with 0.1% crystal violet for 30 sec. The slide was then rinsed with dH_2O, dried and cell morphology observed using a Zeiss Axioskop epifluorescence microscope (Carl Zeiss, Inc. NY). The images were acquired using an AxioCam Mrm camera (Carl Zeiss, Inc. NY). Examination of the cells showed an obvious difference between the AmiB mutant and the wildtype strain in the morphology and arrangement of the cells (Fig. 3). Many cells of the AmiB mutant were altered in shape and some were dramatically increased in cell size (length and width). The AmiB mutant appeared to have a higher percentage of cells in chains. This finding suggests that cell division or septation might be affected. We found no difference in growth rate between wildtype and the AmiB mutant DK630 (data not shown) suggesting that the difference in cell structure is not due to differences in growth rate. While the findings of cells grown on LB plates bred true following subculture, we did not find obvious dramatic differences between the strains when grown in LB broth (data not shown). Although further studies are required to analyze the cellular structure and morphology of the AmiB mutant in more detail, such as using electron microscopy, the results of our studies presented here suggest there is a link between cell division, structure or septation and the rugose phenotype of *V. cholerae*. While we are further studying the role of AmiB, our findings provide evidence for a new function for a prokaryotic amidase, namely its importance in the switch to the rugose phenotype and biofilm formation.

***V. cholerae* RocS: a conserved regulatory protein with a GGDEF and EAL domain regulates the rugose phenotype.** Another class of mutants that we were particularly interested in were mutants with defects in the locus VC0653 encoding a putative protein we have termed RocS (formerly "PdeA-like" protein in the database) containing a GGDEF and EAL domain. It is

important to note that we isolated three independent mutants containing mutations in *rocS* from three independent conjugations. This result suggests that *V. cholerae* RocS has an important role in rEPS production, the rugose phenotype, in biofilm formation and possibly other phenotypes. The defect in the rugose phenotype in this mutant was not explained by differences in growth rate between the wildtype N16961 and RocS (DK567) cells (data not shown). The finding that *V. cholerae* RocS mutants appear to be defective in the switch to the rugose phenotype prompted us to test their motility as described above. Motility assays showed that the RocS mutant (DK567) was consistently >50% reduced in its motility (12 mm zone) compared to its parent N16961 (26 mm zone) suggesting that this locus also affects motility in *V. cholerae* (Fig 2). Based on these results, we propose that *V. cholerae* RocS (and c-di-GMP, see below) regulates several phenotypes, including those with roles in the virulence and the persistence of the species.

Interestingly, the presence of GGDEF domains in all proteins known to be involved in the regulation of cellulose (β-1,4-glucan) synthesis [38]. This is important since cellulose production in *Acetobacter xylinum*, *Rhizobium leguminosarum* bv. *trifolii* and *Agrobacterium tumefaciens* is modulated by the opposing effects of two enzymes, diguanylate cyclase (Dgc) and c-di-GMP diesterase (PdeA), each controlling the level of the novel signaling molecule c-di-GMP in the cell [39-42]. Diguanylate cyclase acts as a positive regulator by catalyzing the formation of c-di-GMP that specifically activates cellulose production while the phosphodiesterase cleaves c-di-GMP and negatively regulates cellulose. The c-di-GMP molecule is predicted to be a reversible, allosteric activator (effector) of cellulose biosynthesis [41]. Furthermore, genetic

complementation studies using genes from different species encoding proteins with GGDEF domains as the only element in common suggest that the GGDEF domain has a role in diguanylate cyclase activity and is important in modulating the level of c-di-GMP [38]. We found that the *V. cholerae* RocS protein plays a key role in the switch to the rugose phenotype that is associated with the production of an EPS-like material and increased biofilm formation.

A BLAST search of the *V. cholerae* RocS shows that it is highly conserved and has significant homologues to putative proteins found in a wide variety of other species including *P. aeruginosa* (PA0575; 42% id; $5e^{-92}$), *Bacillus anthracis* (BA5593; 37% id; $6e^{-90}$), *Ralstonia solanacearum* (RSc0588; 36% id; $4e^{-88}$) and *A. xylinum* (c-di-GMP diguanylate cyclase Dgc; 40%, $9e^{-82}$). Although *dgc* and *pdeA* genes share some homology and have similar domain architecture and we are further studying the role of *V. cholerae* RocS, our finding that the *rocS* mutant is unable to produce an EPS-like material is more consistent with a diguanylate cyclase (*dgc)* mutant that is unable to produce cellulose and *V. cholerae* RocS has slightly higher similarity to *A. xylinum* Dgc than PdeA (data not shown). Recent reports have identified "RocS" homologs in *P. aeruginosa* that appear essential for biofilm formation [43] and in *V. parahaemolyticus* that regulate capsular polysaccharide production [44]. Additionally, the autoaggregation phenotype (which is typical of the rugose phenotype) in the plague bacterium *Y. pestis* requires the GGDEF-containing protein HmsT [45]. Although homologous regulatory (GGDEF-containing) proteins have been found in several species and have been associated with wrinkled colonies, EPS production and biofilm formation, their role in regulating these processes has not been well studied, in part due to the lack of available reagents. There is growing evidence suggesting that GGDEF-containing proteins possess nucleotide cyclase activity [38,46-48] and

are widespread in bacteria [49,50]. The widespread occurrence of this protein homolog and potentially c-di-GMP in prokaryotes suggests a common regulatory system and that they might have an important function in regulating EPS production, the rugose phenotype and biofilm formation in *V. cholerae* and phenotypes in other species. We are currently further investigating the potential role of RocS, its homologues and the signal molecule c-di-GMP in the regulation of various phenotypes.

Conclusion. A rugose-like phenotype has been reported in several species including *S. enterica* Enteritidis [51], *S. enterica* Typhimurium [Anriany, 2001 #2211; [52], *V. parahaemolyticus* [44], *P. aeruginosa* [53] and *Enterobacter sakazakii* [54]. It is now becoming increasingly recognized that the rugose phenotype might have an important role in *V. cholerae* and in several other species suggesting that these "variants" might represent the "tip of an iceberg". Data is accumulating suggesting that rugose variants are filling a specific role in biofilm formation, particular niches or in particular environments. In the case of *V. cholerae*, rEPS production, the rugose phenotype and HFRP may provide some evolutionary or adaptive advantage to that subpopulation of cells in a particular environment.

In the studies reported in this paper, we exploited our identification of conditions that promote the high frequency switch from the smooth to the rugose phenotype of *V. cholerae* to identify and study the genes involved in the molecular switch between the smooth and rugose phenotypes. It appears that some *V. cholerae* strains have evolved an efficient mechanism for a high frequency shift to the rugose phenotype that is associated with EPS production, increased biofilm formation and increased cell survival under specific conditions. The switching

ability that can lead to increased biofilm formation might provide an adaptive advantage in particular environments where strains compete for the same ecological niche and where slight variations in phenotype that promote resistance, increased colonization or cell aggregates are important for adaptation and survival. Our findings of genes involved in the switching process extend our knowledge of the molecular basis underlying the switch between the smooth (EPSoff) and rugose (EPSon) phenotypes of *V. cholerae*.

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Figure 1. Colony morphology of the smooth and rugose variants of *V. cholerae* strain N16961. Panel A, smooth; Panel B, rugose. Colonies are shown following 48 h on LB agar plates.

Figure 2. Swarm plate assay showing the motility of *V. cholerae* strains N16961 (wildtype), AmiB mutant (DK630) and RocS mutant (DK567). Plates contain LB media supplemented with 0.3% agar and were incubated at 37°C for 4 h.

Figure 3. Effect of an AmiB mutation on the cellular morphology of *V. cholerae* strain N16961. Panel A, wildtype cells; panel B, AmiB mutant. Note that the AmiB cells have a difference in morphology and show an increase in overall cell size and the presence of numerous cells in chains. Magnification 1000x.

TABLE 1. Representative HFRP mutants of *V. cholerae* N16961

Mutant[a]	Locus[b]	Predicted protein	Predicted function
DK568 (2)	VC0243	RfbD	LPS biosynthesis, GDP-mannose 4,6 dehydratase
DK623 (1)	VC0244	RfbE	LPS biosynthesis, perosamine synthase
DK578 (2)	VCA0744	GalE	LPS biosynthesis, UDP-glucose 4-epimerase
DK589 (1)	VC0920	Vps (EpsF)	EPS biosynthesis, glycosyl transferase
DK576 (2)	VC0921	Vps (Wzx)	EPS, polysaccharide export, flippase
DK588 (7)	VC0922	Vps	EPS, hypothetical protein
DK562 (13)	VC0665	VpsR	EPS biosynthesis, σ^{54} transcriptional activator
DK614 (10)	VC2628	AroB	aromatic a.a. synthesis, 3-dehydroquinate synthase
DK625 (1)	VC2629	AroK	aromatic a.a. synthesis, shikimate kinase
DK630 (1)	VC0344	AmiB	N-acetylmuramoyl-L-alanine amidase
DK567 (3)	VC0653	RocS	regulatory, contains GGDEF and EAL domains

[a] Numbers in brackets indicate number of mini-Tn5 mutants having insertions in same locus.

[b] Loci and predicted proteins derived from the *V. cholerae* N16961 TIGR sequencing project.

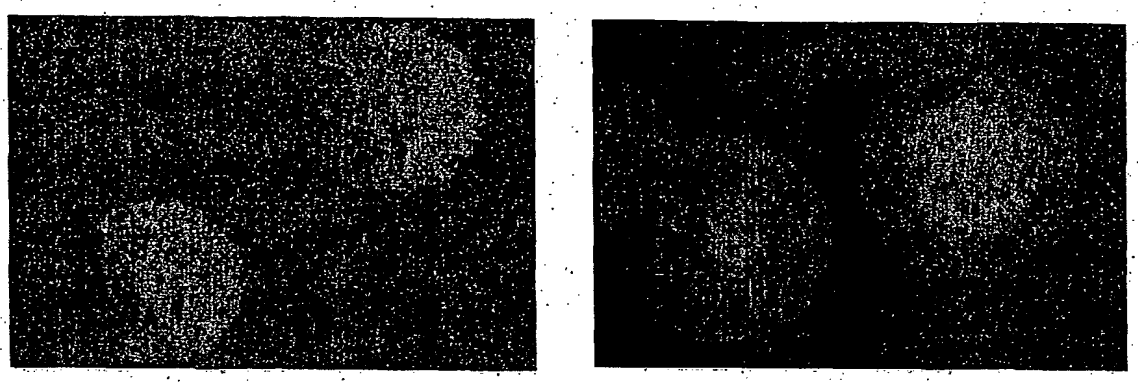


Figure 1. Rashid *et al.* 2003

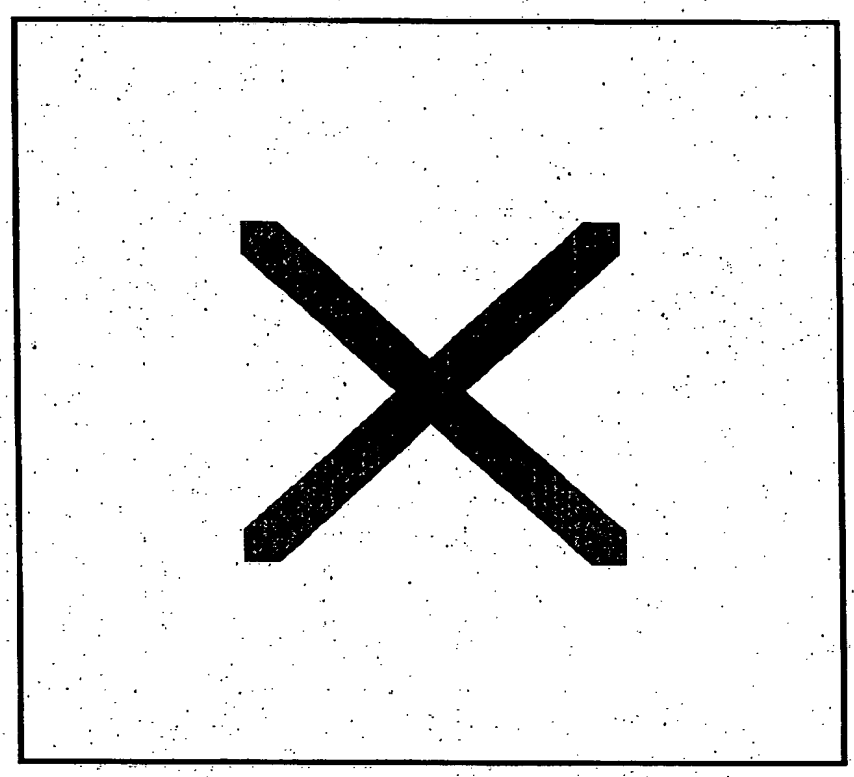


Figure 2. Rashid *et al.* 2003

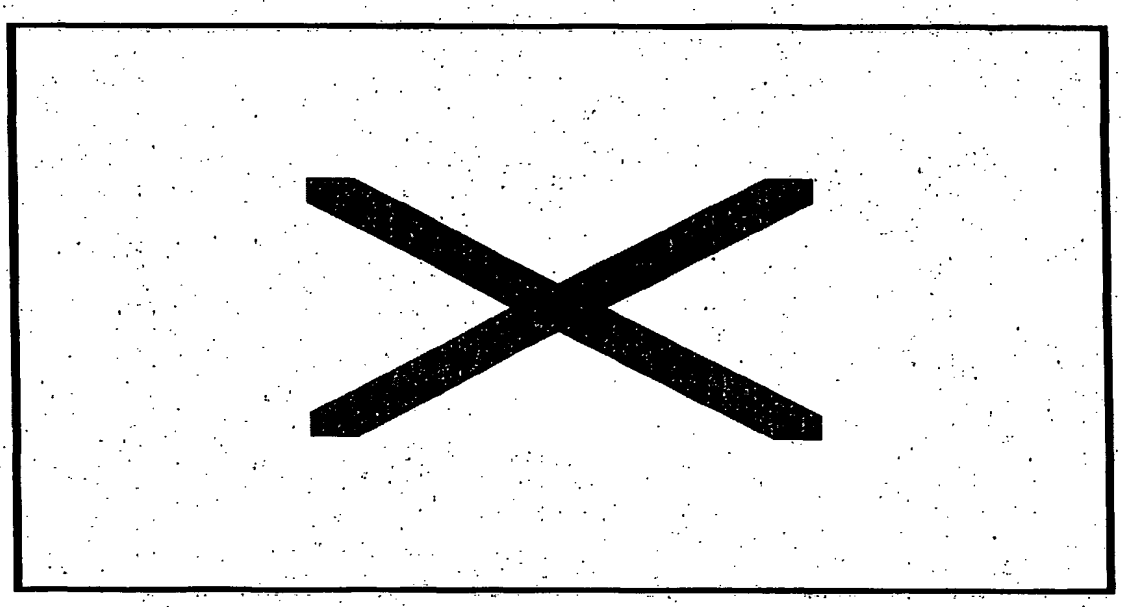


Figure 3. Rashid *et al.* 2003

ABSTRACT

Vibrio cholerae can switch to a "rugose" phenotype characterized by an exopolysaccharide (EPS) matrix, wrinkled colony morphology, increased biofilm formation and increased cell survival under specific conditions. The *vps* gene cluster responsible for the biosynthesis of the rugose EPS (rEPS) is positively regulated by VpsR. We recently identified media (APW#3) promoting EPS production and the rugose phenotype and found that epidemic strains switch at higher frequency than nonpathogenic strains suggesting this switch and extracellular polysaccharide is important in cholera epidemiology. In this study, we performed transposon mutagenesis on the smooth *V. cholerae* strain N16961 to identify mutants that were unable to shift to the rugose phenotype under rugose-inducing conditions to better understand the molecular basis underlying the switch. We identified *vpsR*, *galE* and *vps* genes previously associated with the rugose phenotype, and we also identified proteins not previously associated with the phenotype including *rfbD* and *rfbE* with roles in LPS synthesis and *aroB* and *aroK* with roles in aromatic amino acid synthesis. Additionally, a mutation in *amiB* encoding N-acetylmuramoyl-L-alanine amidase caused defects in the rugose switch, motility and in cell morphology. We also found that a gene encoding a novel regulatory protein we termed RocS (regulation of cell signaling) containing a GGDEF and EAL domain and that is associated with c-di-GMP levels is important for the rugose phenotype, EPS, biofilm formation and motility. We propose that the modulation of cyclic dinucleotide (e.g. c-di-GMP) levels, directly or indirectly, might have potential application in regulating phenotypes of prokaryotic and eukaryotic cells. Our study shows the molecular complexity of the switch between the smooth (EPSoff) and rugose (EPSon) phenotypes of *V. cholerae*.

Background

Cholera and *Vibrio cholerae*

Cholera is an important diarrheal disease of humans that results in significant morbidity and mortality (44, 61). Cholera affects more than 75 countries and every continent (Communicable Disease Surveillance and Response, World Health Organization, www.who.org). A total of 293,121 cholera cases and 10,586 deaths werereported to WHO in 1998 (54). Cholera is acquired by drinking fecally contaminated food or water containing pathogenic *Vibrio cholerae*. Because of its high death-to-case ratio, transmissibility, persistence in the environment and its ability to occur in explosive epidemic form, *V. cholerae* is a public health concern. Furthermore, because of the potential threat of weaponized *V. cholerae* to the food and water supply, it is a priority organism in biodefense research. The threat to the economy, environment and human health is also highlighted by the finding that *V. cholerae* has the potential to be transported internationally and invade new regions through the ballast water of ships (47). *V. cholerae* is known to persist in the environment, however, the factors promoting the environmental persistence of *V. cholerae* are not well understood.

V. cholerae can change its phenotype and reversibly switch from EPSoff (smooth colony morphology) to EPSon (rugose colony morphology) in which the cells are embedded in exopolysaccharide (EPS) and display a wrinkled "rugose" colony morphology and an associated biofilm; (2, 50, 78, 84, 85). Biofilms are the primary mode of existence of most bacterial species and are central to cell survival and persistence (15, 18, 22, 77). The switch to EPSon and the rugose phenotype clearly promotes biofilm formation (50, 63, 78). Importantly, EPS is essential for *V. cholerae* biofilm formation (78). The rugose variant is highly chlorine resistant and shows increasedresistance to killing by acid, UV light and complement-mediatedserum bactericidalactivity (50, 63, 85). Therefore, it has been proposed that switching to EPSon and the rugose phenotype is important in *V. cholerae* and promotes survival in various environments (78, 85). Thus, while the rugose phenotype appeared to be important in *V. cholerae*, studies of *V. cholerae* EPS and the rugose variant were impeded by the low frequency of switching to the rugose phenotype under available conditions. It is now becoming increasingly recognized that the rugose phenotype might have an important role in *V. cholerae* and several other species suggesting that these variants might represent the "tip of an iceberg". The rugose or wrinkled colony phenotype consisting of aggregating cells has been reported in *S. enterica* Enteritidis (59), *S. enterica* Typhimurium, (4), *V. parahaemolyticus* (35), *P. aeruginosa* (57) and *Enterobacter sakazakii* (24). Data is accumulating suggesting that rugose variants are filling a specific role in biofilm formation, particular niches or in particular environments. However, the molecular basis of switching to the rugose phenotype is not well understood.

Importance of Biofilms

Since the introduction of antibiotics, bacterial pathogens have proved remarkably effective at developing resistance. Nosocomial infections result in substantive increases in health care costs, length of hospitalization, and morbidity and mortality (65, 69, 71). Most recently, attention has focused on the emergence of multi-antibiotic resistant pathogens as a cause of such infections; such microorganisms are becoming endemic in U.S. hospitals, further exacerbating already severe problems with nosocomial infections. Gram-negative bacilli are frequently associated with nosocomial infections in ICU patients, particularly ventilator-associated pneumonia and catheter-associated urinary tract infections (Table 1) (27). Of particular concern are nosocomial infections caused by enterobacteria-producing extended-spectrum β-lactamases (ESBLs), particularly *K. pneumoniae*. Organisms that possess these enzymes are usually resistant to multiple antibiotics rendering many currently available potent antimicrobials

useless (64). Evaluation of data from NNIS hospitals shows a dramatic increase in the proportion of *K. pneumoniae* isolates that are resistant to ceftriaxone, cefotaxime, or ceftazidime over the last decade, with a much higher increase particularly among isolates recovered from ICU patients (Fig. 1, Table 2). As such, patients who receive care in ICUs are at increased risk for nosocomial infections, especially pneumonia, urinary tract infection, and bloodstream infection (27). The emergence of antibiotic resistant pathogens in ICUs has made treating infections very difficult, and in some cases, impossible. The duration of stay in the hospital, especially in the ICU, has been associated with the acquisition of ESBL-producing *K. pneumoniae* (12, 60, 70) and has been implicated in inter-facility transmission within a geographic region (49).

TABLE 1. Eight most common pathogens associated with noscomial infection in an ICU patient, National Nosocomial Infections Surveillance System, January 19879-July 1998

	Relative % by Site of Infection				
All sites Pathogen	BSI n=235,758	Pneu n=50,091	UTI n=64,056	SSI n=47,502	n=22,043
S. epidermidis	14.3	39.3	2.5	3.1	13.5
S. aureus	11.4	10.7	16.8	1.6	12.6
P. aeruginosa	9.9	3.0	16.1	10.6	9.2
Enterococci	8.1	10.3	1.9	13.8	14.5
Enterobacter	7.3	4.2	10.7	5.7	8.8
E. coli		7.0	2.9	4.4	18.2 7.1
C. albicans	6.6	4.9	4.0	15.3	4.8
K. pneumoniae	4.7	2.9	6.5	6.1	3.5
Others	30.7	21.8	37.1	25.6	26
Total	100	100	100	100	100

BSI= laboratory confirmed (primary) bloodstream infection; Pneu= pneumonia; UTI= urinary tract infection; SSI= surgical site infection. Adapted from Fridken *et al.* (27).

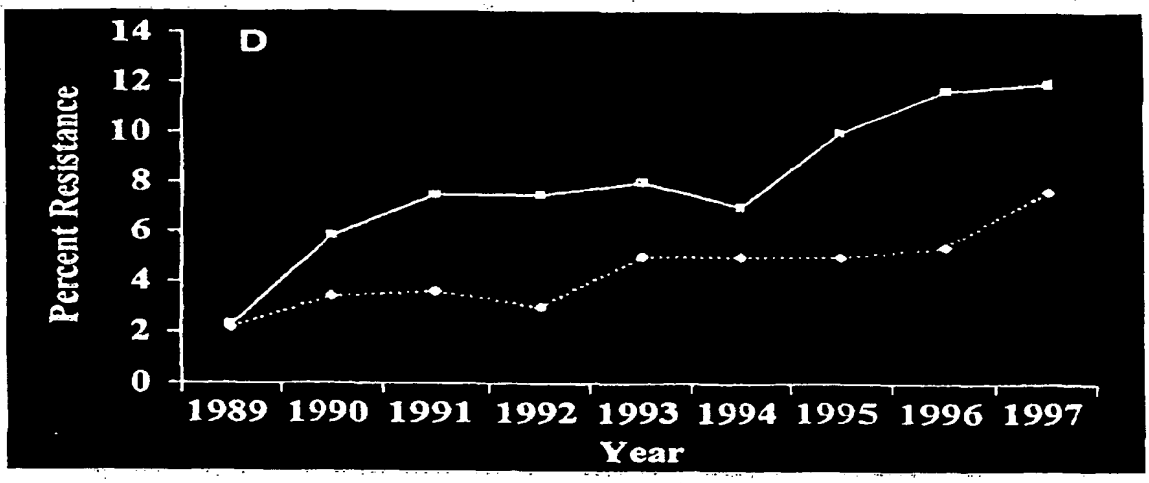


FIGURE 1. Proportion of isolates associated with a nosocomial infection among ICU (solid line) or non-ICU (dotted line) pateinets who were *K. pneumoniae* resistant to third-generation cephalosporins (e.g. ceftriaxone, cefotaxime, or ceftazidime.

TABLE 2. Relative risk of isolating the specific antimicrobial-resistant pathogen from a nosocomial infection occurring in an ICU patient compared with other patients, NNIS, January 1989-1998.

Pathogen	Antimicrobial resistance	Relative Risk among ICU patients (95% CI)*
S. epidermidis	Methicillin	1.22 (1.21-1.24)
S. aureus	Methicillin	1.09 (1.07-1.16)
Enterococci	Vancomycin	1.16 (1.13-1.20)
Enterobacter	Third-generation cephalosprins	1.11 (1.09-1.13)
K. pneumoniae	Third-generation cephalosprins	1.24 (1.20-1.30)
P. aeruginosa	Imipenem	1.16 (1.13-1.21)
P. aeruginosa	Third-generation cephalosprins	1.13 (1.11-1.16)
P. aeruginosa	Ciproflaxacin/ofloxacin	1.03 (1.00-1.05)

* Data from NNIS system, common relative risk and 95% confidence interval, by Mantel-Haenszel Statistic, controlling for year of infection.

Biofilms are the primary mode of existence of many bacterial species and are central to their survival, persistence and often virulence (15, 18, 22, 77). Biofilms resist environmental stresses and adverse conditions better than free-living cells, have increased nutrient availability and can better avoid immune responses (5). A common feature of biofilms is that microorganisms are embedded in an extracellular matrix comprised mostly of EPS (14, 83). EPS is important for the structural and functional integrity of biofilms and determines its physicochemical and biological properties and has a role in adhesion, protection and facilitates community interactions (82). EPS provides protection from a variety of environmental stresses such as UV radiation, pH shifts, osmotic shock, and desiccation.

The role of biofilms in the environmental persistence and transmission of certain pathogens is also well recognized. Like *V. cholerae* (2, 50, 85), *Salmonella enterica* Typhimurium has the ability to form a rugose EPS-producing phenotype which has increased biofilm forming ability and is proposed to have a role in increased persistence in the environment (4). *Salmonella enteritidis* biofilms resistant to cleaning fluids have been shown to persist for at least 4 weeks in domestic toilets after episodes of salmonellosis (10). The finding that *E. coli* and *Salmonella* biofilms can be found on sprouts may make their eradication with antimicrobial compounds difficult and therefore increasing their persistence, resulting in ingestion and infection (25). Alginate EPS production by *P. aeruginosa* protects these strains against chlorine and may contribute to survival of these bacteria in chlorinated water systems (34). The importance of biofilms is also highlighted in the process of horizontal gene transfer since some results suggest that DNA exchange may be increased in bacteria that are attached to a surface and in biofilms rather than between free-swimming planktonic cells (23). This has implications in the transfer of genes encoding functions such as antibiotic resistance or virulence and overall persistence.

Clinically, biofilm formation is known to be a key factor in the establishment and persistence of several difficult to treat infections. Cystic fibrosis is caused by certain *P. aeruginosa* strains which express copious amounts of EPS and form biofilms in the lung (19, 29, 33). The EPS of these *P. aeruginosa* strains makes them

recalcitrant to antimicrobial treatment. Interestingly, like the EPS of *V. cholerae* (2, 50), alginate EPS production by *P. aeruginosa* protects these strains against chlorine and may contribute to survival of these bacteria in chlorinated water systems (34). Another example of a biofilm-mediated infection is chronic ear infection (otitis media) (21). Peridontitis is another example of a biofilm-mediated disease that results from chronic inflammation of the tissue supporting the gums and can lead to tooth loss. The main microbe causing this disease is *Porphyromonas gingivalis* (45). The EPS matrix of biofilms has the potential to physically prevent access of certain antimicrobial agents into the biofilm by acting as an ion exchanger, thereby restricting diffusion of compounds from the external milieu into the biofilm (30, 51, 52). *Helicobacter pylori* produces a biofilm that appears to be important in enhancing resistance to host defense factors and antibiotics and in promoting growth under low pH conditions *in vivo* (72). Biofilm bacteria can be up to 1,000-fold more resistant to antibiotic treatment than the same organism grown planktonically (30). Clinical biofilm infections are marked by symptoms that typically recur even after repeated treatments with antibiotics. Moreover, biofilm infections are rarely resolved by the host's immune system (16). Bacterial biofilms on prosthetic valves are the leading cause of endocarditis in patients who have undergone heart valve replacement. Among patients who develop these infections, the mortality rate is as high as 70% (42). Millions of catheters (e.g., central line, intravenous, and urinary catheters) are inserted into patients every year, and these implants serve as a potential surface for biofilms. Overall, it is thought that upwards of 60% of all nosocomial infections are due to biofilms. These biofilm-based infections can increase hospital stays by up to 2-3 days and cost upwards of $1 billion per year in added costs (6, 7). Unfortunately, the mechanisms leading to EPS production and the development of biofilms are not well understood and additional studies are needed to better understand the role of biofilms in pathogenesis and the signal factors promoting biofilm formation.

Preliminary Data

1. High frequency switching from EPSoff to EPSon in *V. cholerae*

Researchers studying the rugose phenotype of *V. cholerae* (and other species) have been impeded by the very

TABLE 3. Frequency of switching to rugose EPS production (HFRP) by *V. cholerae* strains.

Strains[a]	Serogroup/ Biotype	Source[b]	% Rugose colonies			
			Flask		Tube	
			30°C	37°C	30°C	37°C
N16961	O1/El Tor	C (1971)	24-38	42-51	68-74	60-80
C6709	O1/ElTor	C (1991)	1	23	15	70
NCTC 6585	O1/classical	C (1943)	33-48	44-45	0	0
AMS20A73	O1/classical	C (1945)	3	4	0	0
Aldova	O37	C (1965)	0	1	71-72	23-50
1803	non-O1	C (1992)	0	0	16	
1837	O139	C (1992)	0	0.2	0	0-2
P44	non-O1	E (2000)	12	0	0	2
1085-93	O37	E (1993)	0	0	0.1	0
141-94	O70	E (1994)	0	0	0.3	0
928-93	O6	E (1993)	0	0.2	0.4	0

[a] Listed only are strains showing HFRP or spontaneous rugose colonies.
[b] C, clinical; E, environmental. Year isolated is in parentheses.

low (<1%) frequency of switching between smooth (EPSoff) and rugose cells (EPSon) *in vitro* (50, 75, 80, 81, 85). We have identified culture media and conditions, APW#3 (1% proteose peptone #3, 1% NaCl, pH 8.5.), which results in a high frequency shift of smooth cells (EPSoff) to the rugose phenotype (EPSon). We call this process high frequency rugose production (HFRP) (Table 3 and Fig. 2) (2).

We found that switching to the rugose phenotype at high frequency was more common in epidemic strains than in nonpathogenic strains. We found that 6/19 toxigenic isolates (32%) that were temporally and geographically unrelated and only 1/16 unrelated nontoxigenic strains (6%) could shift to the rugose phenotype (EPSon) and showed HFRP (T test; $P<0.05$) (Table 3). Of all the strains tested, El Tor strain N16961 had the highest switching rates (up to 80%). Reversion, albeit at a lower frequency, from the rugose phenotype to the smooth phenotype was also found showing that phenotypic switching is conditionally transient. These features suggest the switching process might be associated with phase variation-like mechanism. While not all epidemic strains could switch at high frequency, our results showing that switching at high frequency is more correlated with toxigenic strains suggests it is important in *V. cholerae* and suggest a link between this process and virulence. Consistent with previous studies (50), we found a low frequency (<0.5%) shift to the rugose phenotype in several strains. While it is possible that nonpathogenic strains might have to be grown under different conditions to stimulate switching to the EPSon rugose phenotype, this would still nevertheless indicate that there is a difference between clinical and nonpathogenic strains. We found that a sixth pandemic (classical biotype) strain, NCTC 6585, switched at high frequency to the rugose phenotype (up to 48%). HFRP was defined as a >3% shift from the smooth to rugose phenotype (2). To confirm the rugose variant of NCTC 6585 expressed rEPS, we performed transmission electron microscopy (TEM) on ruthenium red stained thin sections. For TEM, 2 day old smooth and rugose colonies on LB agar were removed as 0.5-cm^2 blocks then fixed and stained in a solution of 2% glutaraldehyde, 0.075% ruthenium red, 50 mM lysine monohydrochloride in 0.1 M cacodylate buffer (pH 7.2) for 1 h at room temperature then 18 h at 4°C. Samples were washed twice in 0.1 M cacodylate buffer (pH 7.2), encased in 2% molten Noble agar and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) overnight at 4°C. Samples were then dehydrated in 30-, 50-, 70- and 90% EtOH for 10 min each and twice in 100% EtOH for 15 min each, followed by two treatments with propylene oxide 15 min each then infiltrated using a 1:1 solution of propylene oxide and epon for 2 h at room temperature then in 3:1 epon/propylene oxide overnight. Samples were then placed in pure epon for 1 h, embedded in epon and put in a 60°C oven for 2 days then thin sectioned (50-80 nm thick). Sections were stained with uranyl acetate for 20 min then lead citrate for 20 min. Samples were examined under a JEOL 1200 EX II transmission microscope at 80 kV. TEM of rugose NCTC 6585 showed the presence of extracellular polysaccharide between cells and the absence of this material from smooth cells (Fig. 3). Using rugose-inducing it appears that all major epidemic clones of *V. cholerae* (classical, El Tor and O139) can shift to the rugose phenotype.

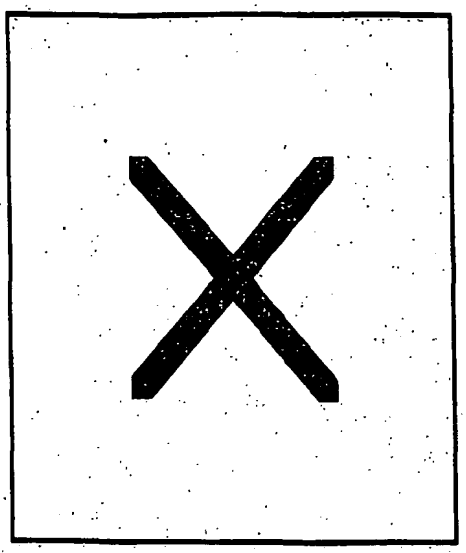
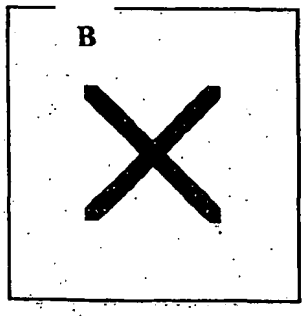
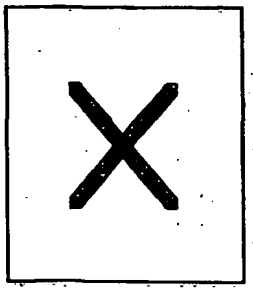
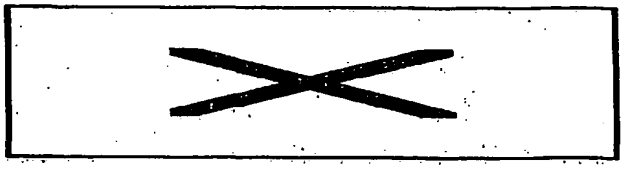


Fig. 2. Colony morphology of smooth and rugose variants of *V. cholerae*. Panel A, colonies at 24 h; Panel B, colonies at 72 h.





promotes high-level resistance



2. The rugose phenotype chlorine

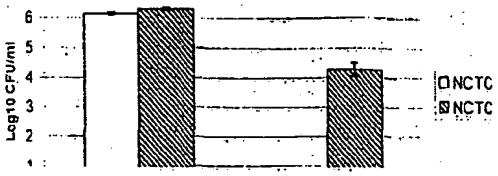


Fig. 4. Effect of chlorine on the su

Production of rEPS is known to promote resistance of El Tor strains to a variety of environmental stresses such as chlorine, UV light, hydrogen peroxide, and complement-mediated bactericidal activity (50, 63, 78, 85). In order to determine whether rugose cells of 6th pandemic classical biotype strain NCTC 6585 promoted resistance to environmental stresses, we exposed smooth and rugose variants to chlorine. Chlorine resistance was assayed (four independent experiments) by using a 1:50 dilution of an overnight culture of NCTC 6585 in 3 ml of fresh LB (Miller) broth. Cultures were then incubated statically at 37°C for 3 h until CFU/ml ~2 x 10^8 CFU/ml, the cells harvested by centrifugation and resuspended in phosphate buffered saline (PBS) (pH 7.2) containing 3 mg/L free chlorine (sodium hypochlorite, Sigma). Following 5 min exposure to 3 mg/L chlorine, cultures were serially diluted and plated on LB agar to determine the number of surviving cells. Consistent with El Tor strain 92A1552 (85), rugose NCTC 6585 cells were 10,000-fold more resistant to chlorine (5 min. exposure to 3 mg/L) than smooth cells (Fig. 4). These findings are the first to report the rugose phenotype by classical biotype strains and shows that rEPS also promotes the survival of classical biotype strains.

3. Switching to EPSon and the rugose phenotype promotes biofilm formation

As the rugose phenotype can promote biofilm formation in El Tor and O139 strains, we tested the ability of smooth and rugose variants of N16961 (El Tor), NCTC6585 (classical) and Aldova (non-O1/non-O139) strains to form biofilms using previously described methods (79). Glass test tubes containing 500 µl LB broth were inoculated with a 1:100 dilution of overnight culture of each variant.

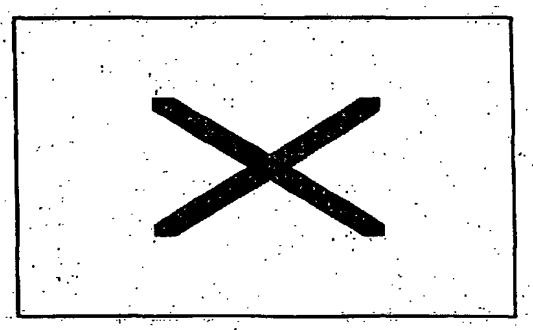


Fig. 5. Biofilm formation by smooth and rugose colony variants of *V. cholerae*.

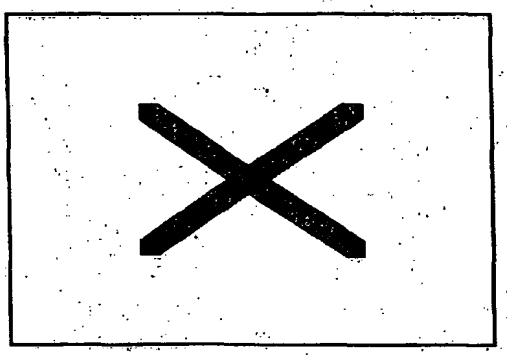


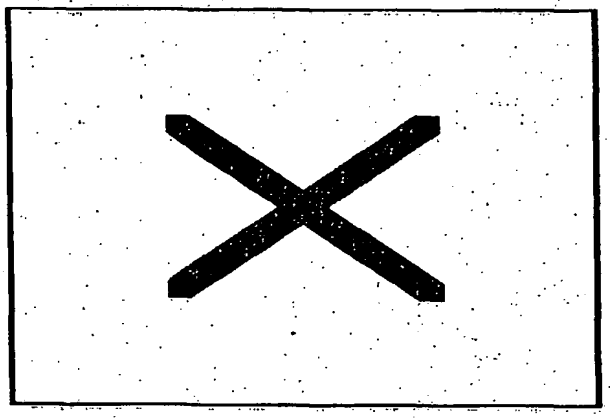
Fig. 6. Biofilm formation in *V. cholerae*. Panel A, NCTC 6585 smooth (no biofilm); panel B, N16961 smooth cels (no biofilm); panel C, NCTC 6585 rugose (biofilm); panel D, N16961 rugose (biofilm).

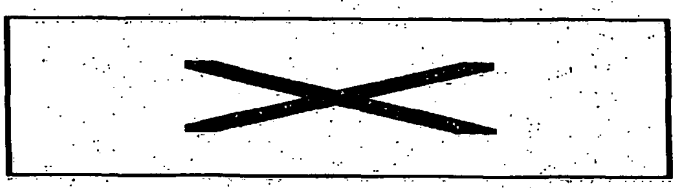
These cultures were then incubated statically at room temperature for 24 h. Culture supernatants were then discarded, tubes rinsed vigorously with distilled water to remove non-adherent cells, filled with 600 µl 0.1% crystal violet (Sigma), incubated for 30 min at room temperature and again rinsed with water. Quantitative biofilm formation was assayed by measuring optical density at 570 nm of the solution produced by extracting cell associated dye with 600 µl DMSO (Sigma). Consistent with other studies (85), the results show that rugose variants of all strains tested had significantly greater (~7-fold) biofilm forming ability than smooth cells (Fig. 5 and 6) and that EPS is essential for *V. cholerae* biofilm formation.

4. *V. cholerae* can switch to the rugose phenotype (EPSon) in the environment

The supposition that switching to EPSon and the rugose phenotype promotes the survival of *V. cholerae* in the environment is based on the premise that switching to EPSon occurs in the environment. However, there have been no reports detecting rugose *V. cholerae* from environmental (or clinical) sources. Unfortunately, there is no current enrichment method for isolating rugose strains from the environment and while TCBS is a selective and differential media for *V. cholerae*, we have found that TCBS inhibits (masks) the rugose phenotype (2).

To test whether smooth cells switch to the rugose phenotype in natural environmental water samples, we used natural lake water from Lake Kittamaqundi that is located on the edge of the City of Columbia, Howard County, Maryland. Lake Kittamaqundi is a 27-acre man-made lake approximately 1 mile long by 1/8th mile wide and has a maximum depth of 7 feet. The Chesapeake Bay, which is approximately only several miles away, is known to be a natural reservoir of *V. cholerae*. We collected fresh water samples from the lake between the warmer months of March-September, 2002. At collection the lake water had pH 7.6 and Na^+ and Cl^- concentrations of 6 mM and 2 mM, respectively. Lake water was autoclaved for 1 h prior to use. In this study, *V. cholerae* strain N16961 was grown in LB broth overnight at 37°C, centrifuged, washed twice with 0.85% NaCl, resuspended in PBS, appropriately diluted and inoculated into 100 ml lake water to a final concentration of 10^4-10^5 cfu/ml confirmed by plate count. Microcosms were incubated statically at room temperature in the dark and at appropriate time intervals aliquots were plated onto LB agar and plate counts and colony morphology were determined. The preliminary results after approx. 6 months sampling suggests that *V. cholerae* N16961 can persist under these conditions with only 1-log decrease in viability. Importantly, N16961 was able to switch from the smooth (EPSoff) to rugose (EPSon) phenotype at high frequency (up to 16%) under these conditions by day 50 (Fig. 7). An advantage of this study is that it closely mimics a true environmental scenario and the switching of a wildtype strain of N16961. While these studies could be extended, these results suggest that *V. cholerae* can shift to the rugose phenotype in natural environments.





5. Compositional analysis of the rEPS in *V. cholerae*

Our previous findings were the first to report the rugose phenotype in a classical (6th pandemic) biotype strain of *V. cholerae*. In order to determine the structural composition of the rugose variant of classical biotype strain NCTC 6585 and to compare it to polysaccharides in other strains, a rugose colony of classical biotype strain NCTC 6585 was inoculated into APW#3 and incubated at 37°C for 3 days under static conditions to promote EPS production and biofilm formation. To harvest the EPS, the cultures were filtered using a large (10 µm) pore size filter (VWR). The biofilm was washed once gently with PBS to remove planktonic cells, transferred to a fresh tube and 3 mm glass beads added to disrupt the biofilm. The sample was then centrifuged at 20,000 rpm (50,000 x g) for 16 h at 4°C to remove cell debris and other contaminants. The supernatant was passed though a Detoxi-Gel Affinitypak column (Polymixin B immobilized on agarose column) (Pierce) to remove any traces of LPS from the sample and DNase and RNase (final conc. 100 µg/ml) was added then incubated at 37°C for 4 h. Proteinase K (final conc. 100 µg/ml) was added and incubated at 37°C overnight followed by 60°C for 15 min. Following the addition of 3 volumes of 95% ethanol, the mixture was precipitated overnight at 4°C then centrifuged at 12,000 rpm for 20 min. The precipitated EPS was washed twice, first with 80% ethanol and then with 95% ethanol. The EPS precipitate was resuspended in 0.5 ml MQ, incubated at –80°C for 2 h, lyophilized for 4 h, then analyzed by combined gas chromatography/mass spectrometry (GC/MS) and performed by the Complex Carbohydrate Research Center in Atlanta, Georgia.

The analysis in Table 4 showed that the rugose EPS (rEPS) for 6th pandemic strain NCTC 6585 differs markedly from the EPS of 7th pandemic strain 92A1552 which has glucose as the predominant sugar (85) and strain TSI-4 which has mannose as the predominant sugar (75). The compositional analysis result also suggests that the extracellular carbohydrate described here is quite different from O1 LPS which typically contains large amounts of perosamine and quinovosamine (62). In contrast to the results of El Tor strain 92A1552 in which 4-linked galactose and 4-linked glucose were the dominant linkages (85), the glycosyl linkage analysis using gas chromatography-mass spectrometry (GC-MS) performed on the classical biotype strain show that the predominant linkage is a 4-linked galactosyl residue and may represent the backbone of the saccharide. Our detailed structural analysis results provide compelling evidence that there are differences in structure of the rEPS and the rugose phenotype between *V. cholerae* strains which can be further studied.

TABLE 4. Glycosyl composition and linkage analysis of rEPS from strain NCTC 6585

Sugar	Glycosyl Composition	Glycosyl linkage		
	%	Glycosyl residue[a]	%	
Rhamnose	8.92	terminal linked –fucosyl residue		9.8
Fucose	10.46	terminal linked –glucosyl residue	7.9	
Mannose	4.68	3 linked –glucosyl residue	8.8	
Galactose	18.71	2 linked –glucosyl residue	15.0	
Glucose	9.29	4 linked –manosyl residue	14.2	
GalNAc	16.86	4 linked –galactosyl residue	24.8	
GlcNAc	27.65	2,3,4 linked –fucosyl residue	7.7	
		2,3 linked –manosyl residue	11.8	

[a] All residues are in the pyranose (p) form.

6. Molecular basis of high frequency switching from EPSoff (smooth) to EPSon (rugose)

Our identification of conditions that promote the switch to the rugose phenotype at high frequency was exploited in the development of a screening assay to identify genes involved in the molecular switch from the smooth to the rugose phenotype. To identify the genes involved in the molecular switch, mini-Tn*5km2* mutagenesis was used (20, 39). Tn5 is contained on the R6K-based plasmid pUT/mini-Tn5 *Km2* (or pUTKm) that is derived from suicide vector pGP704 (48) and can only be maintained in donor strains (e.g. a λ*pir* lysogen of *E. coli*) that produce the R6K-specified λ*pir* protein which is an essential replication protein for R6K and plasmids derived therefrom. It also carries the origin of transfer, *oriT*, of plasmid RP4 which enables efficient conjugal transfer. Delivery of the donor plasmid pUTKm into recipient cells is mediated by the cognate transposase encoded on the plasmid at a site external to the transposon. An advantage of this mutagenesis system is the stability of the Tn5 insertion since the cognate transposase is not carried with the transposon during transposition. Thus, each mutant has only a single Tn5 insertion to screen. In these studies, we mated *E. coli* S17 λpir (pUT/mini-Tn5 Km) with a smooth N16961 (EPSoff) strain. We obtained 14,500 Tn5 mutants from at least 10 independent conjugations and have identified 43 mutants operationally defined as HFRP-negative. These mutants do not produce any detectable rugose colonies under rugose-inducing conditions. To identify the transposon insertion site in these mutants, we used a non-laborious arbitrary primed PCR method followed by DNA sequencing similar to that described previously (9). Briefly, arbitrary PCR was performed in two steps: in the first reaction, chromosomal DNA of the mutant was used as a template for PCR using primers reading out from both sides of the transposon and two arbitrary primers. These primary reactions yielded numerous amplicons including some that were derived from the junction of the transposon insertions. The products of the first-round PCR were purified by Geneclean and amplified using a second pair of outward transposon primers external to the first pair and an arbitrary primer corresponding to the constant region of the original arbitrary primers. This secondary PCR reaction serves specifically to amplify products of the first PCR that include transposon junctions. Amplified fragment ranged between 100- to 800-bp. The products that gave the strongest bands were from agarose gels and sequenced using the same transposon and arbitrary primers used in the second-round PCR. Sequencing was performed using an automated DNA sequencer (model 373A, Applied Biosystems) using the Prism ready reaction dye deoxy termination kit (Applied Biosystems) according to the manufacturer's instructions. We have successfully sequenced and identified the transposon insertion site in the 43 mutants and have performed a BLAST search against the published *V. cholerae* N16961 genome to identify the disrupted genes (36). A summary of these results are shown in Table 5.

TABLE 5. Representative HFRP mutants of *V. cholerae* N16961

Mutant[a]	Locus[b]	Predicted protein	Predicted function
DK568 (2)	VC0243	RfbD	LPS biosynthesis, GDP-mannose 4,6 dehydratase

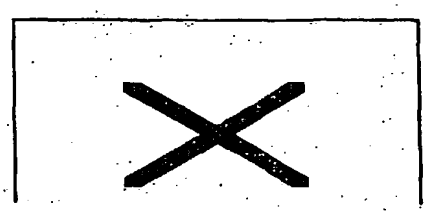
DK623 (1)	VC0244	RfbE	LPS biosynthesis, perosamine synthase
DK578 (2)	VCA0744	GalE	LPS biosynthesis, UDP-glucose 4-epimerase
DK589 (1)	VC0920	Vps (EpsF)	EPS biosynthesis, glycosyl transferase
DK576 (2)	VC0921	Vps (Wzx)	EPS, polysaccharide export
DK588 (7)	VC0922	Vps	EPS, hypothetical protein
DK562 (13)	VC0665	VpsR	EPS biosynthesis, σ^{54} transcriptional activator
DK614 (10)	VC2628	AroB	aromatic a.a. synthesis, 3-dehydroquinate synthase
DK625 (1)	VC2629	AroK	aromatic a.a. synthesis, shikimate kinase
DK630 (1)	VC0344	AmiB	N-acetylmuramoyl-L-alanine amidase
DK567 (3)	VC0653	RocS	regulatory, contains GGDEF and EAL domains

[a] Numbers in brackets indicate number of mini-Tn5 mutants having insertions in same locus.
[b] Loci and predicted proteins derived from the *V. cholerae* N16961 TIGR sequencing project.

Previous transposon mutagenesis studies (including one by our group) have identified gene mutations that result in stable rugose-to-smooth mutants (1, 78, 85). In contrast, taking advantage of our conditions that promote switching to rugose phenotype, we performed transposon mutagenesis on a smooth strain and screened for stable mutants that were unable to switch to the rugose phenotype under rugose-inducing conditions. While our findings revealed mutants with defects in genes previously identified with roles in the rugose phenotype such as several rEPS biosynthesis (*vps* operon) and rEPS regulatory genes (*vpsR)* and LPS genes *(galE)*, our screen identified mutants sustaining insertions in previously unidentified genes. These newly identified mutants could be clustered into several functional groups coding for LPS (*rfbD* and *rfbE*) whereby impairment in the LPS structure might promote shutdown of the rugose (EPSon) phenotype; genes involved in aromatic amino acid synthesis (*aroB* and *aroK*) whereby aromatic amino acid synthesis genes might be directly or indirectly associated with the rugose phenotype; a gene involved in cell wall hydrolysis (*amiB*) and a novel locus, VC0653, designated "*pdeA*-like" in the N16961 genomic database, which we have now termed RocS (for regulation of cell signaling) encoding a putative protein containing GGDEF and EAL domains.

It is important to note that we isolated three independent mutants containing mutations in *rocS* (VC0653) from three independent conjugations. These results suggest that RocS has an important role in rEPS production, the rugose phenotype and in biofilm formation. The defect in the rugose phenotype in the *rocS* mutant was not explained by differences in growth rate between the wildtype (N16961) and RocS (DK567) cells as determine using a spectrophotometer (data not shown). The presence of GGDEF domains in all proteins known to be involved in the regulation of cellulose (β-1,4-glucan) synthesis (8) Cellulose production in *Acetobacter xylinum*, *Rhizobium leguminosarum* bv. *trifolii* and *Agrobacterium tumefaciens* is modulated by the opposing effects of two enzymes, diguanylate cyclase (Dgc) and c-di-GMP phosphodiesterase (PdeA), each controlling the level of c-di-GMP in the cell (Fig. 8) (3, 46, 66, 67). Diguanylate cyclase acts as a positive regulator by catalyzing the formation of c-di-GMP which specifically activates cellulose production while the phosphodiesterase cleaves c-di-GMP and negatively regulates cellulose. c-di-GMP is predicted to be a reversible, allosteric activator (effector) of cellulose biosynthesis (66). Furthermore, genetic complementation studies using genes from different species encoding proteins with GGDEF domains as the only element in common suggest that the GGDEF domain has a role in diguanylate cyclase activity and is important in modulating the level of c-di-GMP (8). We have discovered that this protein homologue regulates EPS production, biofilm formation, motility and presumably other phenotypes.

A BLAST search of the *V. cholerae* RocS shows that it is highly conserved and has significant homologues in a wide variety of other species including *P. aeruginosa* (PA0575; 42% id; $5e^{-92}$), *B. anthracis* (BA5593; 37% id; $6e^{-90}$), *Ralstonia solanacearum* (RSc0588; 36% id; $4e^{-88}$) and *Acetobacter xylinum* (c-di-GMP diguanylate cyclase Dgc; 40%, $9e^{-82}$). Although *dgc* and *pdeA* genes share some homology and have similar domain architecture, our finding that the *V. cholerae* RocS mutant is unable to produce an EPS is more consistent with a *dgc* mutant and RocS has higher similarity with *A. xylinum* Dgc compared to PdeA (data not shown). Recent reports have identified "RocS' homologs in *P. aeruginosa* that appear essential for biofilm formation (13) and in *V. parahaemolyticus* that regulate capsular polysaccharide production (35). Additionally, the autoaggregation phenotype (which is typical of the rugose phenotype) in the plague bacterium *Yersinia pestis* requires the GGDEF-containing protein HmsT (43). Although homologous regulatory GGDEF-containing proteins have been found in several species and have been associated with wrinkled colonies, EPS production or biofilm formation, their role in regulating these processes has not been able to be well studied, in part due to the lack of available reagents. Since there is evidence suggesting that GGDEF-containing proteins possess nucleotide cyclase activity (8,



58, 68, 73) and are widespread in bacteria (17, 28), we propose that modulation of c-di-GMP and its analogs, either directly or indirectly can be used in various applications to regulate prokaryotic and eukaryotic phenotypes.

The bacterial cell wall is typically composed of a heteropolymer known as murein or peptidoglycan. Many Gram-negative bacteria degrade up to 50% of their murein per generation and recycle it to form new murein (31, 32, 55). N-acetylmuramoyl-L-alanine amidases are often associated with autolysis or microbial cell wall hydrolysis. Surprisingly, enzymes in Gram-negatives that cleave the septum such as AmiB have only recently been studied in a few species and in *E. coli*, AmiB mutants are found growing as long chains of unseparated cells (37, 40). In *Azotobacter vinelandii*, an N-acetylmuramoyl-L-alanine amidases is linked to alginate production by the ability of *A. vinelandii* cells to recycle their cell wall (53). Our identification that the AmiB (N-acetylmuramoyl-L-alanine amidase) was linked to switch to the rugose phenotype prompted us to further study the VC0344 locus and its predicted protein.

A BLAST search shows that the *V. cholerae* AmiB sequence has high similarity to N-acetylmuramoyl-L-alanine amidases found in a wide variety of species including *P. aeruginosa* ($7e^{-78}$), *S.* enterica Typhi ($7e^{-69}$), *E. coli* O157:H7 ($6e^{-57}$) and *Y. pestis* ($6e^{-50}$). AmiB in *V. cholerae* is predicted to be a 59-kDa protein that is unusually rich in serine (9.5%), proline (6%) and threonine (6%). Such a composition is common in protein domains associated with the cell wall in Gram-positive bacteria (26) and is similar to a putative peptidoglycan hydrolase of *Lactococcus lactis* (*acmB*) (41). In *V. cholerae*, like *E. coli* and *Y. pestis*, an *amiB* amidase is located immediately upstream of *mutL* which has a role in DNA mismatch-repair (56, 74). A computer analysis using PSORT shows that *V. cholerae* AmiB is predicted to have a cleavable N-terminal signal sequence and an analysis using TMpred strongly predicts that AmiB has two transmembrane domains (score 2363), one at the N-terminal end (a.a. 10-29) which could also represent an N-terminal signal anchor sequence and another transmembrane domain at the C-terminal end (a.a. 446-465). The *V. cholerae* AmiB is predicted to contain a LysM (lysin motif) domain at its C-terminal end and this has been found in enzymes involved in cell wall degradation (11). Interestingly, the *V. cholerae* AmiB contains a Arg-Gly-Asp (RGD) motif that is often associated with a surface binding domain for various mammalian adhesion proteins.

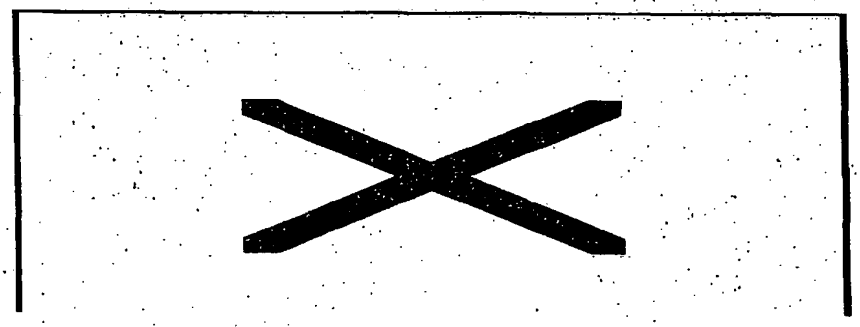


Fig. 9. Effect of an AmiB mutation on the cellular morphology of *V. cholerae* strain N16961. Panel A, wildtype; B, AmiB mutant. Note that the AmiB mutant cells have a difference in morphology and show an increase in overall cell size and the appearance of numerous cells in chains. Both images obtained using 1000x magnification.

Since AmiB has been associated with septation in other species such as E. coli (37, 38), we determined whether the *V. cholerae* AmiB mutant was affected in its cellular morphology as well as the rugose phenotype. In our studies, a single 18 h colony on an LB plate from wildtype N16961 and AmiB mutant DK630 was resuspended in 1 ml PBS and a 50 µl aliquot smeared onto a glass slide, heat fixed then stained with 0.1% crystal violet for 30 sec. The slide was then rinsed with dH_2O, dried and cell morphology observed using a Zeiss Axioskop epifluorescence microscope (Carl Zeiss, Inc. NY). The images were acquired using an AxioCam Mrm camera (Carl Zeiss, Inc. NY). Examination of the cells showed an obvious difference between the AmiB mutant and the wildtype strain in the morphology and arrangement of the cells (Fig. 9). Many cells of the AmiB mutant were altered in shape and some were dramatically increased in cell size (length and width). The AmiB mutant appeared to have a higher percentage of cells in chains. This finding suggests that cell division and septation might be affected. We found no difference in growth rate between the wildtype (N16961) and AmiB mutant (DK630) as determined using a spectrophotometer (data not shown) suggesting that the apparent difference in cell structure is not due to differences in growth rate. While the findings of cells grown on LB plates bred true following subculture, we did not find obvious dramatic differences between the strains when grown in LB broth (data not shown). Although further studies are required to analyze the cellular structure of the AmiB mutant in more detail, such as using electron microscopy, the results of our studies presented here suggest there is a link between cell division, structure or septation and the rugose phenotype of *V. cholerae*. Our findings provide evidence for a new function for a prokaryotic amidase, namely its importance in the switch to the rugose phenotype and biofilm formation.

The importance in regulating *vps* biosynthetic genes in *V. cholerae* led us to further study several *vpsR* transposon mutants, designated DK562 and DK581, respectively. VpsR is encoded by the locus VC0665 and is a 444 amino acid

protein with high similarity to the family of σ54 response regulators such as NtrC, AlgB, and HydG (1, 84). We found that supplying plasmid pDK104 containing *vpsR* on a 2.61-kb PCR fragment which was obtained from strain N16961 using PCR primers KAR486 (5'-CGGGATCCCGCTAAGTCAGAGTTTTTATCGC-3') and KAR487 (5'-TCCCCGCGGGTCGGTGGTTTTGATCGTGT-3'), digested with *Bam*HI and *Sac*II, respectively, and suitably cloned into the low copy vector pWSK29 (76), can restore switching to the rugose phenotype in both these *vpsR* mutants. These findings confirm that the defect in switching to the rugose phenotype in these mutants is due to the mutation in *vpsR*.

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UNIVERSITY OF MARYLAND, BALTIMORE
INVENTION REPORT FORM

NOTE: ALL SECTIONS OF THIS FORM MUST BE COMPLETED.
INCOMPLETE SUBMISSIONS WILL BE RETURNED.

Definition of Invention: An invention is a novel, non-obvious and useful process, machine, article of manufacture, composition of matter, or related improvement. The inventive process consists of two steps: conception (mental formulation of the complete means by which a desired result is achieved) and reduction to practice (physically constructing or carrying out the mental formulation and testing by appropriate means to demonstrate that the invention achieves the desired results).

Definition of Inventor: An inventor is one who contributed to the conception of the invention as well as its reduction to practice. The following are not necessarily co-inventors: co-author, collaborator, co-investigator. In order to be co-inventors, the parties must work toward a common goal, producing an invention by their aggregate efforts. While they need not physically work together, it is necessary that they each work on the same subject matter, each making some contribution to both the inventive thought and to the final result.

1. **Title of Invention:** Methods and Uses of Modulating Microbial Cyclic Dinucleotides

2. **Inventor Information**

Inventor #1. Full Name (printed or typed): David K.R. Karaolis, Ph.D.
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Citizenship: Australia SSN:
Signature: Date: 1/31/03

3. **Advisor approval for student submissions (if applicable):**
Name and Title: Date:

4. **Is this submission related to another invention disclosure previously submitted to ORD?**
Yes ☒ No
If yes, what is the title and/or ORD invention disclosure number of that submission?

5. **Description of Invention:** (NOTE: Use additional sheets if necessary. You may also attach a manuscript, research proposal, drawing, or any other material to support the description below:)

A. In your own words, how would you briefly describe the Invention?

A useful method to regulate the level of biofilm formation, extracellular polysaccharide, virulence and growth of microbial species by modulating the level of c-di-GMP (or analogs). Modulation of c-diGMP levels can be by direct addition of c-di-GMP or by the manipulation of c-di-GMP diguanylates cyclases (and inhibitors) and phosphodiesterases (and inhibitors) whose roles are to synthesize or degrade c-di-GMP levels in the cell, respectively.
Also see attached pages and draft of manuscript.

B. For marketing purposes and federal reporting requirements, please provide a list of five keywords.

c-di-GMP, cyclic dinucleotide, exopolysaccharide, biofilms, colonization, virulence

6. Ownership and Results Demonstrating the Concept is Valid.

A. When was the invention first conceived? Is this date documented? Where?

Email to Andrea Doering 5/28/03

Who employed each co-inventor at that time?
#1. University of Maryland #2.
#3. #4.

B. Has the invention been tested experimentally? Please state if you have preliminary results, animal or laboratory models, prototypes, clinical tests, etc. (Simply reference appropriate sections if you have already included this information. You may also attach additional pages if necessary.)

Regulatory proteins containing GGDEF and EAL domains are highly conserved and found in many prokaryotes and eukaryotic species. Previous studies have provided evidence suggesting that proteins containing a GGDEF domain affect the level of c-di-GMP in cells. The c-di-GMP molecule is a cyclic dinucleotide. The c-di-GMP molecules act as signaling molecules in the cell and have been shown to regulate several important biological processes such as cellulose production in several microbial species. We have recently found that inactivation of a protein (encoded by locus VC0653) containing a GGDEF and EAL domain in the bacterium *V. cholerae* results in a defect in the production of exopolysaccharide, a reduction in biofilm formation ability, and reduces the motility of the bacterium. (see additional pages)

If different from above, who has employed each co-inventor during the period of experimental testing?
#1. As above #2.
#3. #4.

C. Have any of the materials used in the conception and reduction to practice of this invention been acquired from an outside source, for example under a Material Transfer Agreement (excluding outright purchases)?
Yes ☐ No ✠ If yes, please provide details of the transaction below.
Material: Source:
Explanatory Notes: Identify Agreement:

D. Who provided external support for the work that led to the invention? External support includes funding by a nonprofit or for-profit entity of:
(A) an entire research project;
(B) Any portion of a co-inventor's salary or the salary of individuals working under a co-inventor's supervision on a research project; or
(C) supplies, reagents, animals, tissues, cells, or any other materials used in a research project.

1. Entity: Burroughs Wellcome Fund Grant # (if applicable): #1669
Termination date: 8/31/02 Brief explanatory notes: but grant not related to invention
2. Entity: NIH Grant (if applicable): AI-45637
Termination date: Brief explanatory notes: but grant not related to invention

7. **Uses and Applications.** What are the possible uses for the invention? What products could be developed? In addition to immediate applications, are there other uses that might be realized in the future?

Modulation of the levels of c-di-GMP (and analogs) and the use of c-di-GMP cyclase (and inhibitors) or phosphodiesterases (and inhibitors) has potential applications in:

Reducing (or increasing) biofilm formation, reducing (or increasing) exopolysaccharide production, reducing microbial colonization of tissues as well as medical devices and other biotic and abiotic surfaces and reducing biofilm formation. Use as an antimicrobial agent, treatment of infection including in combination with other compounds, modulation of virulence, regulating microbial growth.

8. **Novel Features.** Pick out and expand on the novel and unusual features of the invention. How does it differ from present technology? What problems does it solve? What advantages does it possess?

Regulatory proteins containing GGDEF and EAL domains are highly conserved and found in many prokaryotes and eukaryotic species. These lists are likely to increase with more genomes being sequenced and this sequence information made available. Human pathogen pathogens include: Vibrio cholerae (cholera), Salmonella typhimurium (salmonellosis), Pseudomonas aeruginosa (cystic fibrosis), Bacillus anthracis (anthrax), Yersinia pesitis (plague), Mycobacterium tuberculosis (TB), E. coli O157:H7. Animal pathogens include: Brucella melitensis, Brucella suis. Plant pathogens include: *Agrobacterium tumefaciens* (crown gall tumours), Pseudomonas syringae, Xanthomonas campestris.

There have been no previous reports of showing the use of c-di-GMP and the genes modulating its levels (diguanylate cyclase and associated phosphodiesterase) can affect exopolysaccharide production, biofilm formation and virulence. The ability to affect these phenotypes would be advantageous in preventing and treating acute, persistent and chronic microbial infections.

9. **Obstacles.** Does the invention have any disadvantages or limitations? How can they be overcome?

c-di-GMP is not commercially available

10. **Future Research Plans**

A. What additional research is needed to complete development and testing of the invention?

Synthesis of c-di-GMP (and various analogs) and testing directly their ability to affect biological processes. Tests in animal models.

B. Is this research presently being undertaken? Yes ☐ No ✠

If yes, under whose sponsorship? (but intend to if obtain funding)

Entity: NSF grant application Grant # (if applicable):

Termination date: Brief explanatory notes:

11. **Inventors' Publications/Presentations.**

A. Has the invention or a similar invention in whole or in part been described in a publication? ("Publication" for this purpose includes abstracts of talks, news articles, scientific papers, poster sessions, web postings, thesis, etc.)

Yes ☐ No ✠

B. Has the invention been described orally at meetings or seminars? No

C. Are you planning any disclosure of the invention in the future? What type of disclosure and approximately when?

We plan on publishing our findings (see draft manuscript attached) in ~6 months.

PLEASE NOTE: In order to complete review of your invention report and timely filing of a patent application, if appropriate, TEC-COM may require up to 60 days before any future publication date.

12. Prior Art

A. A literature search should be done by the inventor to determine publications relevant to this invention. Please list publications and any related patents you may know of. Use additional sheets if necessary. Free patent searches can be performed on-line using the IBM searcher (http://www.patents.ibm.com/) or the USPTO home page (http://www.uspto.gov.pat/index.html). Free Medline literature searches can be performed using the PubMed searcher (http://www.ncbi.nlm/nih.gov/PubMed/). Please attach a print-out of relevant hits.
See attached documents.

B. Do you know of relevant information presented at a public talk, trade fair, sales catalogue? Has the invention or a similar product been used publicly or has it been offered for sale?

Other investigators has found that mutations in bacterial proteins containing GGDEF domains alters the cells ability to form biofilms and in some cases produce cellulose.

13. Marketing Information

A. Has there been any commercial interest in this invention? Please name companies and specific contacts.
Invention has not been disclosed.

B. What companies do you think may be interested in this invention? Why?

Pharmaceuticals with interests in preventing and treating microbial infections and reducing microbial colonization of tissues, surfaces, medical devices.

C. What companies make products currently in the market that compete with your invention?
None.

D. What is the potential size of the market? For example, if the invention is a new therapeutic agent, can you give an estimate of the number of people afflicted in the U.S. and abroad?
60% of all hospital-acquired infections are due to biofilm-associated microbes. The infections alone cost $4 billion per year in added costs. This does not include non-hospital acquired infections so the potential size of the market is large.

E. If your invention would be primarily used in countries other than the U.S., please suggest which countries and give a brief explanation.
Worldwide

F. What other research groups are working on similar inventions and where are they located?
Other Investigators at Harvard University, Dartmouth University and many other institutions are working on ways to control biofilms. This is a HOT field of study.

Please note: If ORD pursues patent protection based on this invention report, it will be necessary to have a commercial licensee identified within approximately 18 months of patent application filing.

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Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse